



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> METHOD FOR GENERATING A SUBTRACTED cDNA LIBRARY AND USES OF THE GENERATED LIBRARY		
<b>(57) Abstract</b> <p>This invention provides a method of generating a subtracted cDNA library of a cell comprising: a) generating a cDNA library of the cell; b) isolating double-stranded DNAs from the cDNA library; c) releasing the double-stranded cDNA inserts from the double-stranded DNAs; d) denaturing the isolated double-stranded cDNA inserts; e) hybridizing the denatured double-stranded cDNA inserts with a labelled single-stranded nucleic acid molecules which are to be subtracted from the cDNA library; and f) separating the hybridized labeled single-stranded nucleic acid molecule from the double-stranded cDNA inserts, thereby generating a subtracted cDNA library of a cell. This invention also provides different uses of the subtracted library.</p>		

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-1-

**METHOD FOR GENERATING A SUBTRACTED cDNA LIBRARY AND USES  
OF THE GENERATED LIBRARY**

5 This application is a continuation-in-part of United States Application Serial No. 08/143,576 filed October 27, 1993, the contents of which are hereby incorporated by reference.

10 The invention described herein was supported in part by National Cancer Institute grants CA35675 and CA43208. The United States Government has certain rights in this invention.

15 **Background of the Invention**

Throughout this application, various publications are referenced by within parentheses. Full citations for these publications may be found at the end of each series  
20 of experiments. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed therein.

25 Malignant melanoma is increasing at a rapid rate in North American populations and it is estimated that 1 in 100 children currently born may eventually develop superficial spreading-type melanoma (1). Although  
30 readily curable at early stages, surgical and chemotherapeutic intervention are virtually ineffective in preventing metastatic disease and death in patients with advanced states of malignant melanoma (1). These observations emphasize the need for improved therapeutic  
35 approaches to more efficaciously treat metastatic

-2-

melanoma. A potentially useful therapeutic modality for this and other malignancies could involve the use of agents capable of inducing an irreversible loss in proliferative capacity in tumor cells without the requirement for direct cytotoxicity, that is, the differentiation therapy of cancer (2-5). In previous studies, applicants have demonstrated that it is possible to reprogram human melanoma cells to undergo terminal cell differentiation with a concomitant loss of proliferative capacity by treatment with the combination of recombinant human fibroblast interferon (IFN- $\beta$ ) plus the antileukemic compound mezerein (MEZ) (6,7). The combination of IFN- $\beta$  + MEZ induces terminal differentiation in melanoma cells, innately resistant to the antiproliferative effect of either agent alone, and in human melanoma cells selected for resistance to growth suppression induced by IFN- $\beta$  (6,7). In contrast, treatment with IFN- $\beta$  or MEZ alone results in the development of specific components of the differentiation program in human melanoma cells, but these agents do not induce most melanoma cells to undergo terminal cell differentiation (6-8).

Terminal differentiation induced by IFN- $\beta$  plus MEZ in human melanoma cells is associated with an increase in melanin synthesis, changes in cellular morphology (characterized by the production of dendrite-like processes), modifications in cell surface antigenic profile, and an irreversible loss of proliferative capacity (3, 6-10). When used separately, IFN- $\beta$  and MEZ induce both growth suppression and increased melanin synthesis and MEZ induces the production of dendrite-like processes in specific human melanoma cells (6,8). Trans-retinoic acid (RA) is effective in inducing tyrosinase activity and enhancing melanin synthesis in specific

-3-

human melanoma cultures without altering cell growth, whereas mycophenolic acid (MPA) can induce growth suppression, increased tyrosinase activity and melanin synthesis, and dendrite formation (11). In contrast, the combination of IFN- $\beta$  + recombinant immune interferon (IFN- $\gamma$ ) results in a synergistic suppression in the growth of human melanoma cells without inducing enhanced melanin synthesis or morphologic changes (10,12). These observations suggest that the various changes induced during the process of differentiation in human melanoma cells, that is, increased tyrosinase activity and melanin synthesis, antigenic changes, dendrite formation, and growth suppression, can occur with and without the induction of terminal cell differentiation.

An unresolved issue is the nature of the gene expression changes that occur in human melanoma cells reversibly committed to differentiation vs. human melanoma cells irreversibly committed to terminal differentiation. This information will be important in defining on a molecular level the critical gene regulatory pathways involved in growth and differentiation in human melanoma cells. To begin to address these questions, applicants have used various experimental protocols that result in either growth suppression without the induction of differentiation-associated properties, a reversible induction of differentiation-associated traits, or terminal cell differentiation in the H0-1 human melanoma cell line. As potential target genes relevant to these processes, applicants have analyzed early growth response, extracellular matrix, extracellular matrix receptor, and interferon-responsive genes. No unique gene expression change was observed solely in H0-1 cells induced to terminally differentiate vs. cultures reversibly growth arrested. However, treatment of H0-1

-4-

cells with IFN- $\beta$  + MEZ was associated with specific patterns of gene expression changes that were also apparent in H0-1 cells cultured in conditioned medium obtained from terminal-differentiation-inducer-treated

5 H0-1 cells. Exposure to either the terminal differentiation-inducing compounds or -conditioned medium resulted in the enhanced expression of HLA Class I antigen, melanoma growth stimulatory activity (gro-MGSA),

10 interferon-stimulated gene-15 (ISG-15), and fibronectin. These observations support the potential involvement of a type I interferon and a gro/MGSA autocrine loop in the chemical induction of differentiation in H0-1 cells

-5-

**Summary of the Invention**

This invention provides a method of generating a subtracted cDNA library of a cell comprising: a) generating a cDNA library of the cell; b) isolating double-stranded DNAs from the cDNA library; c) releasing the double-stranded cDNA inserts from the double-stranded DNAs; d) denaturing the isolated double-stranded cDNA inserts; e) hybridizing the denatured double-stranded cDNA inserts with a labelled single-stranded nucleic acid molecules which are to be subtracted from the cDNA library; and f) separating the hybridized labeled single-stranded nucleic acid molecule from the double-stranded cDNA inserts, thereby generating a subtracted cDNA library of a cell. The invention also provides different uses of the generated library.

This invention also provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene. This invention further provides different uses of the isolated melanoma differentiation genes.

## Brief Description of the Figures

Fig. 1A-B      Effect of continuous exposure to various growth and differentiation modulating agents on the 96-h growth of the human melanoma cell line, H0-1. Cells were seeded at  $5 \times 10^4$ /35-mm tissue culture plate and 24 h later the medium was changed with the indicated compounds. Cell numbers were determined by Coulter Counter after 96-h growth. Further details can be found in Materials and Methods. The abbreviations and the concentrations of test compounds uses: IFN- $\beta$  + MEZ (recombinant human fibroblast interferon + mezerein) (2,000 U/ml + 10 ng/ml); MPA + MEZ (mycophenolic acid + MEZ) ( $3.0 \mu\text{M}$  + 10 ng/ml); RA + MEZ (trans-retinoic acid + MEZ) ( $2.5 \mu\text{M}$  + 10 ng/ml); IFN- $\beta$  + IFN- $\gamma$  (IFN- $\beta$  + recombinant human gamma interferon) (1,000 U/ml + 1,000 U/ml); MEZ (10 ng/ml); MPA ( $3.0 \mu\text{M}$ ); IFN- $\gamma$  (2,000 U/ml); IFN- $\beta$  (2,000 U/ml); RA ( $2.5 \mu\text{M}$ ); and Control (media only).

Fig. 2      Effect of growth and differentiation modulating agents on steady-state c-jun, jun-B, c-myc, HLA Class I antigen, HLA Class II (DR $\beta$ ), ISG-54, ISG-15, gro/MGSA, and GAPDH mRNA levels in H0-1 cells. Total RNA was isolated 96 h after treatment with the various agents. The concentrations of compounds used were the same as those used for growth studies (see

-7-

legend to Figure 1). Ten micrograms of total cytoplasmic RNA was electrophoresed, transferred to nylon filters, and hybridized with the indicated <sup>32</sup>P-labeled gene probes. Further details can be found in Materials and Methods of the first series of experiments.

Fig. 3

Effect on gene expression in H0-1 cells of continuous and transient exposure to agents inducing a reversible commitment to differentiation or terminal cell differentiation. In the left panel [24h (+)], H0-1 cells were treated with IFN- $\beta$  + MEZ (2,000 U/ml + 10 ng/ml), MPA + MEZ (3.0  $\mu$ M + 10 ng/ml), RA + MEZ (2.5  $\mu$ M + 10 ng/ml), or MEZ (50) (50 ng/ml) for 24 h. In the right panel [24 h (+) 72 h (-)], H0-1 cells were treated with the same test agents used in the left panel for 24 h, the cells were washed three times in serum-free medium and cells were cultured for an additional 72 h in DMEM-10 in the absence of test compound. Total RNA was isolated, electrophoresed, transferred to nylon filters, and hybridized with the indicated <sup>32</sup>P-labeled gene probes. Further details can be found in Materials and Methods of the first series of experiments.

Fig. 4

Effect of growth and differentiation modulating agents on steady-state  $\alpha_5$  integrin,  $\beta_1$  integrin, fibronectin,  $\beta$ -

-8-

5                    actin,  $\gamma$ -actin, tenascin, and GAPDH mRNA  
                    levels in H0-1 cells. Total RNA was  
                    isolated 96 h after treatment with the  
                    various agents. The concentrations of  
10                   compounds used were the same as those used  
                    for growth studies (see legend to Figure  
                    1). Ten micrograms of total RNA was  
                    electrophoresed, transferred to nylon  
                    filters, and hybridized with the indicated  
15                    $^{32}$ P-labeled gene probes. Further details  
                    can be found in Materials and Methods of  
                    the first series of experiments.

15                   Fig. 5                   Effect on gene expression in H0-1 cells of  
                    continuous and transient exposure to  
                    agents, inducing a reversible commitment  
                    to differentiation or terminal cell  
                    differentiation. Experimental details are  
20                   described in the legend to Figure 3 and in  
                    Materials and Methods of the first series  
                    of experiments.

25                   Fig. 6                   Effect of conditioned medium from H0-1  
                    cells treated with agents, resulting in a  
                    reversible commitment to differentiation  
                    or terminal cell differentiation on gene  
                    expression in naive H0-1 cells. H0-1  
                    cells were untreated (Control) or treated  
30                   for 24 h with IFN- $\beta$  + MEZ (2,000 U/ml + 10  
                    ng/ml), MPA + MEZ (3.0  $\mu$ M + 10 ng/ml), RA  
                    + MEZ (2.5  $\mu$ M + 10 ng/ml), or MEZ (50) (50  
                    ng/ml). The medium was then removed, the  
                    cultures were washed three times with  
                    serum-free medium, and grown for an



-9-

5 additional 72 h in complete growth medium. The conditioned medium was then collected, and contaminating cells were removed by centrifugation. Conditioned medium was mixed with an equal volume of DMEM-10 (1:2), left panel CONDITIONED MEDIUM (1:2) (24 h), and applied to previously untreated (naive) H0-1 cells for 24 h. Alternatively, conditioned medium was mixed with three parts of DMEM-10 (1:4), right panel CONDITIONED MEDIUM (1:4) (96 h), and applied to previously untreated (naive) H0-1 cells for 96 h. Total RNA was isolated and analyzed by Northern blotting with the DNA probes indicated. Further details can be found in Materials and Methods of the first series of experiments.

20 Fig. 7

Effect of conditioned medium from H0-1 cells treated with agents, resulting in a reversible commitment to differentiation or terminal cell differentiation on extracellular matrix, extracellular matrix receptor, and cytoskeletal gene expression in naive H0-1 cells. Experimental details are as described in the legend to Figure 6 and in Materials and Methods of the first series of experiments.

30

Fig. 8

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Flowchart for constructing a subtractive differentiation inducer treated human melanoma cell cDNA library. cDNA libraries were constructed from differentiation inducer [IFN- $\beta$  (2000

-10-

units/ml) + MEZ (10 ng/ml)] treated human melanoma (H0-1) cells ( $Ind^+$ ) and untreated control ( $Ind^-$ ) H0-1 cells. Using the protocols outlined, an H0-1 IFN- $\beta$  + MEZ ( $Ind^+$ ) subtracted cDNA library was constructed.

Fig. 9

Determination of the purity of the single-stranded DNA and double-stranded DNA preparations of cDNA libraries. Photograph of a 1% agarose electrophoresis gel stained with ethidium bromide and photographed under illumination with UV light. The single-stranded DNA was prepared from the control ( $Ind^-$ ) library and double-stranded DNA from IFN- $\beta$  + MEZ ( $Ind^+$ ) library. Lane M,  $\lambda$  HindIII molecular weight markers; lane 1, single-stranded DNA; lane 2, single-stranded DNA digested with EcoRI and XhoI; lane 3, double-stranded DNA; lane 4, double-stranded DNA digested with EcoRI and XhoI.

Fig. 10

Northern blot analyses of untreated control and IFN- $\beta$ , MEZ and IFN- $\beta$  + MEZ treated H0-1 cells probed with cDNA clones isolated from an H0-1 IFN- $\beta$  + MEZ ( $Ind^+$ ) subtracted cDNA library. RNA was isolated from cells untreated or treated for 24 hours with IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml) or IFN- $\beta$  + MEZ (2000 units/ml and 10 ng/ml). Ten micrograms of total cellular RNA were separated on a 1% agarose gel, transferred to nylon

-11-

membranes and then hybridized individually with radioactive probes prepared using appropriate inserts from the cDNA clones. The series of cDNAs isolated from inducer treated H0-1 cells have been tentatively called *mda*, melanoma differentiation associated, genes. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a control for uniform loading and expression of the RNA samples.

Fig. 11

Homology of *mda-3* to the cDNA of human macrophage inflammatory protein (GOS19-1). The DNA sequence of *mda-3* was obtained using the Sanger dideoxynucleotide sequencing method. For sequence homology with known genes, the DNA sequence of *mda-3* was compared using the GCG/FASTA program and the GenBank/EMBL database. The top sequence corresponds to the *mda-3* cDNA and the bottom sequence corresponds to GOS19-1.

Fig. 12

Effect of IFN- $\beta$  and MEZ, alone and in combination on the growth of H0-1 human melanoma cells (see third series of experiments for further details).

Fig. 13

Effect of IFN- $\beta$  and MEZ, alone and in combination on the morphology of H0-1 human melanoma cells. Cells were treated with 2,000 units/ml of IFN- $\beta$ , 10 ng/ml MEZ or the combination of agents for 24 h.

Fig. 14

Expression of proliferative sensitive

-12-

proteins (P2Ps) in human melanoma cells treated with growth suppressing and differentiation inducing compounds. The combination of IFN- $\beta$  + MEZ induces terminal differentiation in F0-1 human melanoma cells, but not in SV40-transformed human melanocytes. Experimental details for determining P2Ps can be found in Minoo et al. (52) and Witte and Scott (53) of the third series of experiments.

Fig. 15

Northern blot analyses of untreated and treated H0-1 cells probed with cDNA clones isolated from an H0-1 IFN- $\beta$  + MEZ (Ind<sup>+</sup>) subtracted cDNA library. RNA was isolated from cells treated for 96 h with the agents indicated. Concentration of agents were the same as used in Fig. 1. Experimental details can be found in Jiang and Fisher (49) and Jiang et al. (14) of the third series of experiments. IFN- $\beta$  2,000 units/ml; IFN- $\lambda$  2,000 units/ml; MEZ 10 ng/ml; MPA 3 $\mu$ M; RA 2.5 $\mu$ M; IFN- $\beta$  + IFN- $\gamma$  (1,000 units/ml of each IFN); IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml); MPA + MEZ (3 $\mu$ M + 10 ng/ml); RA + MEZ (2.5  $\mu$ M + 10 ng/ml).

Fig. 16

Expression of mda genes in human melanomas. - = control, + = IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml).

Fig. 17

Expression of mda genes in normal

-13-

cerebellum, glioblastoma multiforme and normal skin fibroblasts. - = control, + = IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml).

## 5      Fig. 18

Expression of mda genes in human colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) and prostate carcinoma (LNCaP). - = control, + = IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml).

10

## Fig. 19

Effect of various treatment protocols on mda expression in H0-1 cells. IFN- $\beta$  (2,000 units/ml, 24 hours); MEZ (10 ng/ml, 24 hours); IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml, 24 hours); phenyl butyrate (PB) (4mM, 24 hours, 4d, 7d);  $\gamma$  Rad ( $\gamma$  irradiation) 3 gray, 24 hours, Act D (actinomycin D) (5  $\mu$ g/ml, 2 hour  $\rightarrow$  24 hours assay); Adriamycin (Adr) (0.1  $\mu$ g/ml, 24 hours); Vincristine (Vin) (0.1  $\mu$ g/ml, 24 hours); cis-plt (cis-platinum) (0.1  $\mu$ g/ml, 24 hours); TNF- $\alpha$  (tumor necrosis factor -  $\alpha$ ) (100 units/ml, 24 hours); UV (10 joules/mm<sup>2</sup>, 2, 14 and 24 hours assay); VP-16 (5  $\mu$ g/ml, 24 hours); IFN- $\alpha$  (2,000 units/ml, 24 hours); and IFN- $\alpha$  + MEZ (2,000 units/ml + 10 ng/ml, 24 hours).

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20

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## Fig. 20.

Stages in the development of metastatic melanoma.

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## Fig. 21A-E.

Effect of recombinant human IFN- $\beta$  and mezerein (MEZ) used alone and in combination on the morphology of H0-1 and

-14-

B0-2 human melanoma cells (X150). (A) Control H0-1 cells 24 h postplating. (B) H0-1 cells exposed to 2,000 units/ml IFN- $\beta$  for 24 h. (C) H0-1 cells exposed to 10 ng/ml MEZ for 24 h. (D) H0-1 cells exposed to 2,000 units/ml IFN- $\beta$  and 10 ng/ml MEZ for 24 h. (E) Control B0-2 cells 24 h postplating. (F) B0-2 cells exposed to 2,000 units/ml IFN- $\beta$  for 24 h. (G) B0-2 cells exposed to 10 ng/ml MEZ for 24 h. (H) B0-2 cells exposed to 2000 units/ml IFN- $\beta$  and 10 ng/ml MEZ for 24 h. Data from reference [18] of the fifth series of experiments.

Fig. 22

Components of the differentiation process in H0-1 human melanoma cells. Abbreviations: IFN- $\beta$ : recombinant human fibroblast interferon; IFN- $\gamma$ : recombinant immune interferon; MPA: mycophenolic acid; MEZ: mezerein; Trans RA: trans retinoic acid. Relative growth suppression: 4+ = ~80% reduction in growth in comparison with untreated control cultures; 3+ = ~50 to 60% reduction in growth in comparison with untreated control cultures; 2+ = ~40% reduction in growth in comparison with untreated control cultures; 1+ = ~30% reduction in growth comparison with untreated control cultures.

Fig. 23A-B

cDNA and predicted amino acid sequence of *mda-6*. The predicted translation begins at nucleotide 95 and ends at nucleotide 589. Accession number U09579 (GeneBank).

-15-

Fig. 24

Cellular changes mediating enhanced expression of certain *mda* genes and differential expression of particular *mda* genes during tumor progression and in normal versus tumor-derived cell types. Specific *mda* genes have been identified that display enhanced expression during treatment with agents that induce growth suppression, DNA damage (including chemotherapeutic agents that function by different mechanisms) and/or terminal differentiation. Additional *mda* genes have also been shown to display differential expression as a function of tumor progression and in matched sets of normal versus tumor-derived human cells.

Fig. 25A-B

Induction of *mda-6* (WAF1/CIP1/SDI1) expression in HL-60 cells by TPA and RA. Total cytoplasmic RNA was isolated from HL-60 cells treated with TPA (3 nM) for .25, .5, 1, 2, 4 or 6 d and from HL-60 cells treated with RA (1  $\mu$ M) for .5, 1, 2, 4 or 6 d. A 10- $\mu$ g aliquot of RNA was run on a 1.0% agarose gel and transferred to a nylon filter. Blots were hybridized with a multi-prime  $^{32}$ P-labeled *mda-6* gene probe. Filters were stripped and rehybridized with a multiprimer  $^{32}$ P-labeled GAPDH probe.

Fig. 26A-C

Early induction of *mda-6* (WAF1/CIP1/SDI1) expression in HL-60 and TPA-resistant HL-60 (HL-525) cells treated with TPA, RA and

-16-

Vit D3. Cells were treated with TPA (3 nM), RA (1  $\mu$ M) or Vit D3 (400 nM) for 1, 2, 3, 6 or 12 h. RNA was isolated and analyzed by RT-PCR using appropriate *mda-6* or GAPDH specific primers as described in the Materials and methods.

## Fig. 27A-C

Induction of *mda-6* (WAF1/CIP1/SDI1) expression in HL-60 and TPA-resistant HL-60 (HL-525) cells after extended incubation with TPA, RA and Vit D3. Cells were treated with TPA (3 nM), RA (1  $\mu$ M) or Vit D3 (400 nM) for .5, 1, 2, 4 or 6 d. RNA was isolated and analyzed by RT-PCR using appropriate *mda-6* or GAPDH specific primers as described in the Materials and methods.

## Fig. 28

Induction of the MDA-6 (WAF1/CIP1/SDI1) encoded protein p21 in HL-60 cells treated with TPA, DMSO and RA. Lysates from untreated HL-60 (control) and HL-60 cells treated with TPA (3 nM), DMSO (1%) or RA (1  $\mu$ M) for 12, 24, 48 and 72 h were immunoprecipitated with WAF1/CIP1 (MDA-6) polyclonal antibody or actin monoclonal antibody as described in Materials and methods. The size of the MDA-6 protein is 21 kDa and the size of the Actin protein is 42 kDa.

## Fig. 29A-B

Effect of CHX on the induction of *mda-6* in HL-60 and TPA-resistant HL-60 (HL-525) cells. Cells were treated with CHX (10  $\mu$ g/ml) for 1, 3, 6 or 10 h or with CHX (10



-17-

5  $\mu$ g/ml) plus TPA (3 nM) for 1+, 3+, 6+ or 10+ h. RNA was isolated and analyzed by RT-PCR using appropriate *mda-6* or GAPDH specific primers as described in the Materials and methods in the sixth series of experiments.

## Fig. 30

10 Open-reading frame of *mda-6*. Predicted translation of the *mda-6* cDNA begins at nucleotide 95 and ends a nucleotide 589. Accession number U09579 (Genbank). *mda-6* encodes a 164 amino acid protein with an M of 21,000 that is identical to the cyclin dependent kinase inhibitor, p21.

## Fig. 31A-E

15 Induction of *mda-6* (p21) expression in H0-1 human melanoma cells as a function of differentiation and growth suppression. H0-1 cells were untreated (control) or treated for 24 h (panel A), 96 h (panel B) or treated for 24 h followed by growth for 72 h in the absence of inducer (panel C) with IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml) or IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml). In panel D, H0-1 cells were grown for the indicated time in medium containing 5% fetal bovine serum (Control) or without fetal bovine serum (DMEM-0). In panel E, high-density H0-1 cells were incubated in DMEM-0 for the times indicated. RNA isolation, Northern blotting and hybridization with *mda-6* and GAPDH was performed as described (Jiang & Fisher, 1993; Jiang et al., 1993).

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-18-

Fig. 32

Effect of IFN- $\beta$  + MEZ on mda-6 (p21) expression in human melanoma cells and an SV40-transformed human melanocyte culture. High-density melanoma cells (H0-1, F0-1, L0-1, SH-1, WM239 and WM278) and logarithmically growing low-density SV40-transformed human melanocytes (SV516-SV) were treated with IFN- $\beta$  (2000 units/ml) + MEZ (10 ng/ml) for 24 hr, total RNA was isolated and analyzed by Northern blotting (Jiang & Fisher, 1993; Jiang et al., 1993). Filters were probed with mda-6 then stripped and probed with GAPDH as described (Jiang & Fisher, 1993; Jiang et al., 1993).

Fig. 33

Effect of 24 h treatment of H0-1 cells with IFN- $\beta$ , MEZ and IFN- $\beta$  + MEZ on p53 and p21 levels. Lysates from untreated (control) and H0-1 cells treated with IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml) or IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml) for 24 h were immunoprecipitated with p53 monoclonal antibody Ab1 (PAb421), p21 (WAF1/CIP1) polyclonal antibody or an actin monoclonal antibody as described in Materials and methods in the seventh series of experiments.

Fig. 34

Expression of p53 and p21 as a function of growth arrest and terminal differentiation in H0-1 cells. Lysates from untreated (control) and H0-1 cells treated with IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml) or IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml) for 48,

-19-

72 and 96 h were immunoprecipitated with p53 monoclonal antibody Ab1 (PAb421), p21 (WAF1/CIP1) polyclonal antibody or an actin monoclonal antibody as described in Materials and methods in the seventh series of experiments.

Fig. 35

Relative expression of *mda-6* during melanoma progression. The level of *mda-6* relative to GAPDH was determined by quantitative (comparative) RT-PCR in actively growing cells. RGP: radial growth phase primary melanoma. VGP: vertical growth phase primary melanoma. Results represent average for: 5 melanocyte cultures, one dysplastic nevus culture, one SV40-transformed immortalized melanocyte culture, one RGP and four early VGP primary melanomas and six metastatic melanomas.

Fig. 36

Expression of *mda-6* (p21) as a function of Matrigel-mediated progression in primary human melanomas. The level of *mda-6* and GAPDH was determined by RT-PCR for the indicated actively proliferating cell lines. P1 and P2 refers to the first and second passage, respectively, through nude mice of RGP or early VGP melanoma cells in combination with Matrigel.

Fig. 37

Northern blot analysis of *mda-6* (p21) expression in C8161 human melanoma and chromosome 6 containing C8161 human melanoma subclones. Levels of *mda-6* and

-20-

GAPDH mRNAs were determined in actively growing untreated cells (-) and cells treated for 96 hr with IFN- $\beta$  + MEZ (1000 units/ml + 10 ng/ml) as described (Jiang & Fisher, 1993; Jiang et al., 1994a).

5

## Fig. 38A-B

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Nucleotide sequence and deduced encoded amino acid sequence of the *mda-7* cDNA. The hydrophobic putative transmembrane domain and three potential N-glycosylation residues are underlined; the 3' untranslated sequence contains three putative instability motifs ATTA (underlined) and two consensus signals AATAAA for poly(A) addition (underlined).

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## Fig. 39

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Properties and structure of the *mda-7* encoded gene product as predicted by GCG/Plotstructure program.

## Fig. 40

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Hydrophobic analysis and structural predictions of the *mda-7* encoded gene product by GCG/Peplot program. A potential transmembrane domain is predicted using this program.

## Fig. 41

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## Fig. 42

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Structure of *mda-7* encoded gene produce as predicted by the Chou-Fosman method.

Relative expression of *mda-7* during melanoma progression. The level of *mda-7* relative to GAPDH was determined quantitative (comparative) RT-PCR in actively growing cells. The cell lines examined were (1) normal melanocytes; (2)

-21-

primary melanoma cells, including RGP and VGP cells; and (3) metastatic melanoma cells.

5      Fig. 43

10      Expression of *mda-6* and *mda-7* as a function of aging and senescence in human fibroblast cells. IMR90 cells analyzed at late passage (OLD) versus early passage (YOUNG). IDH4 cells are IMR90 cells  
15      immortalized by an SV40 T-antigen (transcriptionally controlled by a dexamethasone (DEX) inducible mouse mammary tumor virus promoter). Growth of IDH4 cells in DEX results in active growth and expression of an immortalized  
20      phenotype (a YOUNG IMR90 phenotype). In contrast, removal of DEX results in a shutdown of the T-antigen and loss of proliferative capacity and senescence (SENESCENT). RNA was extracted from the four cell lines and analyzed by reverse  
25      transcription-polymerase chain reaction (RT-PCR) using primer sequences specific for *mda-6*, *mda-7* or GAPDH. As a control for expression of these genes, RNA from H0-1 human melanoma cells treated with  
30      IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml) for 96 hr was used. H0-1 cells under these conditions are irreversibly growth arrested and terminally differentiated.

-22-

## Detailed Description of the Invention

Throughout this application, the following standard abbreviations are used to indicate specific nucleotides:

5           C=cytosine           A=adenosine  
          T=thymidine         G=guanosine

10           This invention provides a method of generating a subtracted cDNA library of a cell comprising: a) generating a cDNA library of the cell; b) isolating double-stranded DNAs from the cDNA library; c) releasing the double-stranded cDNA inserts from the double-stranded DNAs; d) denaturing the isolated double-stranded cDNA inserts; e) hybridizing the denatured double-stranded  
15   cDNA inserts with a labelled single-stranded nucleic acid molecules which are to be subtracted from the cDNA library; and f) separating the hybridized labeled single-stranded nucleic acid molecule from the double-stranded cDNA inserts, thereby generating a subtracted cDNA  
20   library of a cell.

In an embodiment, the cDNA library of the cell is a  $\lambda$ ZAP cDNA library.

25   This invention provides the above-described method, wherein the releasing of the double-stranded cDNA is performed by digestion with appropriate restriction enzymes.

30   This invention also provides the above-method of generating a subtracted cDNA library of a cell, wherein the denaturing of step d) is by boiling.

35   In an embodiment, the single-stranded nucleic acid molecules are DNAs. In a further embodiment, the single-

-23-

stranded nucleic acid molecules are labelled with biotin.  
In a still further embodiment, the single-stranded  
nucleic acid molecules are labelled with biotin, wherein  
the separating of step f) is performed by extraction with  
5 streptavidin-phenol: Chloroform.

Other methods for labelling single-stranded nucleic acid  
molecules are well-known in the art.

10 This invention further provides the above-described  
methods of generating a subtracted cDNA library of a  
cell, wherein the single-stranded nucleic acid molecules  
are from another cDNA library. In another embodiment,  
this cDNA library is a  $\lambda$ ZAP cDNA library.

15 In another embodiment, the single-stranded nucleic acid  
molecules are from another cDNA library, wherein the cDNA  
library is a  $\lambda$ ZAP cDNA library, wherein the cell is an  
HO-1 melanoma cell treated with IFN- $\beta$  and MEZ.

20 In another embodiment, wherein the cDNA library is a  $\lambda$ ZAP  
cDNA library, the cell is an HO-1 melanoma cell treated  
with IFN- $\beta$  and MEZ and the single-stranded nucleic acid  
molecules are from another cDNA library of a HO-1  
25 melanoma cell.

In still another embodiment of the above-described  
methods of generating a subtracted cDNA library of a  
cell, wherein the cDNA library is a  $\lambda$ ZAP cDNA library,  
30 wherein the cell is terminally differentiated and the  
single-stranded nucleic acid molecules are from another  
cDNA library of an undifferentiated cell.

In still another embodiment of the above-described  
35 methods of generating a subtracted cDNA library of a

-24-

cell, wherein the cDNA library is a  $\lambda$ ZAP cDNA library, wherein the cell is undifferentiated and the single-stranded nucleic acid molecules are from another cDNA library of a terminally differentiated cell.

5

In one embodiment of the above-described method of generating a subtracted cDNA library of a cell, the cell is selected from a group consisting essentially of neuroblastoma cell, glioblastoma multiforme cell, myeloid  
10 leukemic cell, breast carcinoma cell, colon carcinoma cell, endometrial carcinoma cell, lung carcinoma cell, ovarian carcinoma cell and prostate carcinoma cell.

15

In another embodiment of the above-described method of generating a subtracted cDNA library of a cell, the cell is induced to undergo reversible growth arrest or DNA damage and the single-stranded nucleic acid molecules are from another cDNA library of an uninduced cell.

20

In still another embodiment of the above-described method of generating a subtracted cDNA library of a cell, the cell is at one developmental stage and the single-stranded nucleic acid molecules are from another cDNA library from the cell at different developmental stage.

25

In still another embodiment of the above-described method of generating a subtracted cDNA library of a cell, the cell is cancerous and the single-stranded nucleic acid molecules are from another cDNA library from a normal  
30 cell.

35

In another embodiment, the cell is from the breast, brain, meninges, spinal cord, colon, endometrium, lung, prostate and ovary.



-25-

This invention also provides a method of generating a subtracted cDNA library of a cell comprising: a) generating a cDNA library of the cell; b) isolating double-stranded DNAs from the cDNA library; c) releasing  
5 the double-stranded cDNA inserts from the double-stranded DNAs; d) denaturing the isolated double-stranded cDNA inserts; e) hybridizing the denatured double-stranded cDNA inserts with a labelled single-stranded nucleic acid molecules which are to be subtracted from the cDNA  
10 library; and f) separating the hybridized labeled single-stranded nucleic acid molecule from the double-stranded cDNA inserts, thereby generating a subtracted cDNA library of a cell, wherein the single-stranded nucleic acid molecules are from another cDNA library, wherein the  
15 cDNA library is a  $\lambda$ ZAP cDNA library, further comprising introducing the subtracted library into host cells.

This invention provides a subtracted library generated by the method generating a subtracted cDNA library of a cell  
20 comprising: a) generating a cDNA library of the cell; b) isolating double-stranded DNAs from the cDNA library; c) releasing the double-stranded cDNA inserts from the double-stranded DNAs; d) denaturing the isolated double-stranded cDNA inserts; e) hybridizing the denatured  
25 double-stranded cDNA inserts with a labelled single-stranded nucleic acid molecules which are to be subtracted from the cDNA library; and f) separating the hybridized labeled single-stranded nucleic acid molecule from the double-stranded cDNA inserts, thereby generating  
30 a subtracted cDNA library of a cell.

This invention provides a subtracted library generated by the method of generating a subtracted cDNA library of a cell comprising: a) generating a cDNA library of the  
35 cell; b) isolating double-stranded DNAs from the cDNA

-26-

library; c) releasing the double-stranded cDNA inserts from the double-stranded DNAs; d) denaturing the isolated double-stranded cDNA inserts; e) hybridizing the denatured double-stranded cDNA inserts with a labelled single-stranded nucleic acid molecules which are to be subtracted from the cDNA library; and f) separating the hybridized labeled single-stranded nucleic acid molecule from the double-stranded cDNA inserts, thereby generating a subtracted cDNA library of a cell, wherein the single-stranded nucleic acid molecules are from another cDNA library, wherein the cDNA library is a  $\lambda$ ZAP cDNA library, wherein the cell is an HO-1 melanoma cell treated with IFN- $\beta$  and MEZ, wherein the single-stranded nucleic acid molecules are from another cDNA library of a HO-1 melanoma cell.

This invention provides a method of identifying a melanoma differentiation associated gene comprising: a) generating probes from clones of the above-described subtracted library; and b) hybridizing the probe with total RNAs or mRNAs from HO-1 cells treated with IFN- $\beta$  and MEZ and total RNAs or mRNAs from untreated HO-1 cells, hybridization of the probe with the mRNAs from the treated HO-1 cell but no or reduced hybridization with the total RNAs or mRNAs from untreated cells indicating that the clone from which the probe is generated carries a melanoma differentiation associated gene.

This invention further provides a melanoma differentiation associated gene identified by the above method.

This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of *mda-4* gene.

-27-

This invention further provides a method of detecting the expression of *mda-4* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecule capable of specifically hybridizing with a sequence of *mda-4* gene under conditions permitting hybrid formation; and c) detecting hybrids formed, the detection of the hybrids indicating expression of *mda-4* gene in the cell.

This invention also provides a method to indicate the tissue lineage of a cell comprising detecting the expression of *mda-4* gene using the above-described method, the expression of *mda-4* gene indicating that the tissue lineage of the cell is neuroectodermal.

This invention also provides a method for distinguishing a fibroblast or epithelial cell from a melanoma or central nervous system cell comprising detecting the expression of *mda-4* gene using the above-described method, the expression of *mda-4* indicating that the cell is a melanoma cell or a central nervous system lineage cell.

This invention provides a method to monitor the response to DNA damage induced by gamma irradiation and UV irradiation of a cell comprising hybridizing the nucleic acid molecule capable of specifically hybridizing with a sequence of *mda-4* gene, hybridization of the nucleic acids from the cell indicating that there is a response to the DNA damage of the cell.

This invention provides a method of monitoring a response to treatment with chemotherapeutic agents which work in a similar manner as cis-platinum in a cell comprising hybridizing the nucleic acids from the cell with the

-28-

nucleic acid molecule capable of specifically hybridizing with a sequence of *mda-4* gene, hybridization of the nucleic acids from the cell responds to the treatment with the chemotherapeutic agents.

5

This invention further provides a method for detecting types I or II interferons in a sample comprising: a) incubating the sample with the 5' regulatory element of the *mda-4* gene under conditions permitting binding of  
10 types I and II interferons transcriptional regulatory proteins to the regulatory elements; and b) detecting the binding of types I or II interferons transcriptional regulatory proteins to the regulatory elements, the binding indicating the presence of type I or II  
15 interferons in the sample.

As used herein, the term "sample" is broadly defined. It includes, but not limited to bodily fluids such as urine, saliva, blood and other clinical samples.

20

Transcriptional regulatory proteins which are responsive to type I or II interferons are well-known in the art.

In an embodiment, the target cell is a eukaryotic cell.  
25 In a separate embodiment, the 5' regulatory element is linked to the native *mda-4* gene and the detection of binding is by examination of the elevated expression of the *mda-4* gene. In an embodiment, the 5' regulatory element is linked to a marker gene. In a further  
30 embodiment, the marker gene is  $\beta$ -galactosidase or CAT.

This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-1*. In an embodiment, the  
35 nucleic acid is a cDNA. In another embodiment, the

-29-

nucleic acid is genomic DNA.

In a separate embodiment, the *mda-1* gene is coding for a human protein.

5

This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-2*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the

10

In a separate embodiment, the *mda-2* gene is coding for a human protein.

15

This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-4*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

20

In a separate embodiment, the *mda-4* gene is coding for a human protein.

25

This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-5*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

30

In a separate embodiment, the *mda-5* gene is coding for a human protein.

This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing

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with a sequence of the nucleic acid molecule, *mda-5*.

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This invention provides a method of detecting the expression of *mda-5* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule *mda-5* under conditions permitting hybrids formation; and c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-5* gene in the cell.

This invention provides a method for distinguishing a normal neuroectodermal cell from a malignant neuroectodermal cell comprising detecting the expression of *mda-5* gene using the method of detecting the expression of *mda-5* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule *mda-5* under conditions permitting hybrids formation; and c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-5* gene in the cell, the expression of *mda-5* gene indicating that the cell is normal neuroectodermal cell.

This invention provides a method for detecting types I or II interferons in a sample comprising: a) incubating the sample with the 5' regulatory element of the *mda-5* gene under conditions permitting binding of types I and II interferons transcriptional regulatory proteins to the regulatory elements; and b) detecting the binding of the types I or II interferons transcriptional regulatory proteins to the regulatory elements, the binding indicating the presence of type I or type II interferons

-31-

in the sample.

5 In an embodiment, the cell is a eukaryotic cell. In another embodiment, the 5' regulatory element is linked to the native *mda-5* gene and the detection of binding is by the examination of the elevated expression of *mda-5* gene.

10 In a separate embodiment, the 5' regulatory element is linked to a marker gene. In a further embodiment, the marker gene is  $\beta$ -galactosidase, luciferase or CAT.

15 This invention provides a method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising: a) incubating the human melanoma cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-5* using the above-described method, the expression of *mda-5* gene indicating that the compound is capable of inducing terminal differentiation in human melanoma cells.

20 This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-6*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

25 In a separate embodiment, the *mda-6* gene is coding for a human protein.

30 This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule, an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-*

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-32-

6.

This invention provides a method of detecting the expression of *mda-6* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule, an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-6* under conditions permitting hybrids formation; and c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-6* gene in the cell.

This invention provides a method for distinguishing a normal neuroectodermal cell from a malignant neuroectodermal cell comprising detecting the expression of *mda-6* gene using the method of detecting the expression of *mda-6* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules capable of recognizing the *mda-6* gene under conditions permitting hybrids formation; and c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-6* gene in the cell, the expression of *mda-6* gene indicating that the cell is normal neuroectodermal cell.

30

The invention also provides a method for distinguishing an adenocarcinoma cell from a carcinoma cell comprising detecting the expression of *mda-6* gene using the above-described method, the expression of *mda-6* gene indicating that the cell is a carcinoma cell.

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-33-

The invention further provides a method for monitoring the response of a cell to an anticancer agent such as actinomycin-D or adriamycin comprising detecting the expression of *mda-6* gene using the above-described method, the expression of *mda-6* gene indicating that the cell responds to the anticancer agent.

This invention provides a method for monitoring response to topoisomerase inhibitor by a cell comprising detecting the expression of *mda-6* using the above-described method of detecting the expression of *mda-6* gene in a cell, the expression of *mda-6* indicating that the cell responds to the topoisomerase inhibitor.

This invention provides a method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising: a) incubating the human melanoma cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-6* in a cell using the above-described method, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human melanoma cells.

This invention provides a method for identifying a compound capable of inducing terminal differentiation in human leukemia cells comprising a) incubating the human leukemia cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-6* using the above-described method, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human leukemia cells.

This invention also provides a method for identifying a compound capable of inducing terminal differentiation in

-34-

human lymphoma cells comprising: a) incubating the human lymphoma cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-6* using the above-described method, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human lymphoma cells.

This invention provides a method for identifying a compound capable of inducing terminal differentiation in human neuroblastoma cells comprising: a) incubating the human neuroblastoma cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-6* using the above-described method, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human neuroblastoma cells.

This invention also provides a method for identifying a compound capable of inducing terminal differentiation in human glioblastoma multiforme cells comprising: a) incubating the human glioblastoma cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-6* using the above-described method, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human glioblastoma multiforme cells.

This invention also provides a method for distinguishing an early stage from a more progressed human melanoma cell comprising detecting the expression of *mda-6* gene using the above-described method, the expression of *mda-6* gene indicating that the cell is a less progressed human melanoma cell.

This invention provides a method for reversing the

-35-

5 malignant phenotype of cells comprising: (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; and (b) introducing the linked *mda-6* gene into the malignant cells for the expression of the *mda-6* gene, thereby reversing the malignant phenotype of cells.

10 This invention also provides a method for reversing the malignant phenotype of cells comprising: (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; (b) introducing the linked *mda-6* gene into the malignant cells; and (c) placing the cells from  
15 step (b) in appropriate conditions to express the *mda-6* gene such that the expression of the *mda-6* gene will reverse the transforming phenotype of the malignant cells.

20 This invention also provides a method of reversing the phenotype of malignant cells in a subject comprising:  
(a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; (b) introducing the  
25 linked *mda-6* gene into the malignant cells for the expression of the *mda-6* gene, thereby reversing the phenotype of the malignant cells.

30 This invention also provides a method of reversing the phenotype of malignant cells in a subject comprising:  
(a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; (b) introducing the  
linked *mda-6* gene into the malignant cells of the  
35 subject; and (c) inducing the expression of the *mda-6*

-36-

gene which will reverse the transforming properties of the cells, thereby reversing the phenotype of the malignant cells in the subject.

5 This invention also provides a method of inducing growth suppression in tumorigenic and metastatic cells comprising: (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; (b)  
10 introducing the linked *mda-6* gene into the tumorigenic and metastatic cells; and (c) inducing the expression of the *mda-6* gene, thereby inducing growth suppression in tumorigenic and metastatic cells.

15 This invention also provides a method of inducing terminal differentiation in tumorigenic and metastatic cells comprising: (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; (b)  
20 introducing the linked *mda-6* gene into the tumorigenic and metastatic cells; and (c) inducing the expression of the *mda-6* gene, thereby inducing terminal differentiation in tumorigenic and metastatic cells.

25 In an embodiment, the cell is a melanoma, leukemia, lymphoma, neuroblastoma, glioblastoma or carcinoma cell.

In a separate embodiment, the regulatory element is a promoter. In a further embodiment, the promoter is a  
30 tissue-specific promoter. In another embodiment, the promoter is an inducible promoter.

The linked *mda-6* gene may be introduced into the cells by naked DNA technology, retroviral vectors, antibody-coated  
35 liposomes, mechanical or electrical means. These

-37-

technologies are known in the art.

This invention provides a method of determining the stage of a melanoma comprising: (a) obtaining appropriate  
5 amount of cells from the melanoma; (b) measuring the expression level of the *mda-6* gene in the cells; and (b) comparing the expression level with predetermined standards of normal and melanoma cells in different stages, thereby determining the stage of a melanoma.

10

In an embodiment, the expression is measured by the antibodies against the *mda-6* protein. In another embodiment, the expression is measured by in situ hybridization.

15

This invention also provides a method for indicating the effectiveness of a treatment against cancer comprising measuring the expression level of *mda-6* gene in the cells of the cancer, the increase of the expression level  
20 indicating the effectiveness of the treatment. In an embodiment, the cancer is melanoma. In another embodiment, the cancer is leukemia. In a separate embodiment, the cancer is lymphoma. In another embodiment, the cancer is neuroblastoma. In a further  
25 embodiment, the cancer is a glioblastoma multiforme tumor. In a still further embodiment, the cancer is a carcinoma.

This invention provides an isolated nucleic acid molecule  
30 encoding a protein produced by a melanoma differentiation associated gene designated *mda-7*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

35 In a separate embodiment, the *mda-7* gene is coding for a

-38-

human protein.

This invention provides an isolated nucleic acid molecule of a cDNA, wherein the protein is a human protein.

5

This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of *mda-7*.

10

This invention provides a method of detecting the expression of *mda-7* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-7* under conditions permitting hybrids formation; and c) detecting hybrids

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formed, the detection of the hybrids formed indicating the expression of *mda-7* gene in the cell.

This invention provides a method for determining whether a cell is a melanoma cell or a carcinoma cell comprising detecting the expression of *mda-7* gene using the above-described method of detecting the expression of *mda-7* gene in a cell, the expression of *mda-7* gene indicating that the cell is a melanoma cell.

25

30

The invention further provides a method for distinguishing a melanocyte or early stage melanoma cell from an advanced metastatic melanoma cell comprising detecting the expression of *mda-7* gene using the above-described method, the expression of *mda-7* gene indicating that the cell is a melanocyte or early stage melanoma

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-39-

cell.

5 This invention provides a method for distinguishing a normal neuroectodermal cell from a malignant neuroectodermal cell comprising detecting the expression of *mda-7* gene using the above-described method of detecting the expression of *mda-7* gene in a cell, the expression of *mda-7* gene indicating that the tissue lineage of the cell is normal neuroectodermal cell.

10

The invention also provides a method for distinguishing a fibroblast from an epithelial cell comprising detecting the expression of *mda-7* gene using the above-described method, the expression of *mda-7* gene indicating that the cell is a fibroblast.

15

20 This invention provides a method for identifying a compound capable of inducing growth suppression in human melanoma cells comprising: a) incubating appropriate concentration of the human melanoma cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-7* using the above-described method, the expression of *mda-7* gene indicating that the compound is capable of inducing growth suppression in human melanoma cells.

25

30 The invention further provides a method for monitoring the response of a cell to an anticancer agent such as adriamycin or vincristine comprising detecting the expression of *mda-7* gene using the above-described method, the expression of *mda-7* gene indicating that the cell responds to the anticancer agent.

35 The invention also provides a method for monitoring the response of a cell to DNA damage induced by UV

-40-

irradiation comprising detecting the expression of *mda-7* gene using the above-described method, the expression of *mda-7* gene indicating that the cell responds to the DNA damage.

5

This invention provides a method for reversing the malignant phenotype of cells comprising: (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; and (b) introducing the linked *mda-7* gene into the malignant cells for the expression of the *mda-7* gene, thereby reversing the malignant phenotype of cells.

15

This invention also provides a method for reversing the malignant phenotype of cells comprising: (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; (b) introducing the linked *mda-7* gene into the malignant cells; and (c) placing the cells from step (b) in appropriate conditions to express the *mda-7* gene such that the expression of the *mda-7* gene will reverse the transforming phenotype of the malignant cells.

25

This invention also provides a method for reversing the phenotype of malignant cells in a subject comprising: (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; and (b) introducing the linked *mda-7* gene into the malignant cells for the expression of the *mda-7* gene, thereby reversing the phenotype of the malignant cells.

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This invention also provides a method to reversing the



-41-

phenotype of malignant cells in a subject comprising:

(a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; (b) introducing the linked *mda-7* gene into the malignant cells of the subject; and (c) inducing the expression of the *mda-7* gene which will reverse the transforming properties of the cells, thereby reversing the phenotype of the malignant cells in the subject.

10

This invention also provides a method of inducing growth suppression in tumorigenic and metastatic cells comprising: (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; (b) introducing the linked *mda-7* gene into the tumorigenic and metastatic cells; and (c) inducing the expression of the *mda-7* gene, thereby inducing growth suppression in tumorigenic and metastatic cells.

20

This invention also provides a method of inducing terminal differentiation in tumorigenic and metastatic cells comprising: (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; (b) introducing the linked *mda-7* gene into the tumorigenic and metastatic cells; and (c) inducing the expression of the *mda-7* gene, thereby inducing terminal differentiation in tumorigenic and metastatic cells. In an embodiment, the cell is a melanoma cell. In another embodiment, the cell is a leukemia cell. In a further embodiment, the cell is a lymphoma cell. In a still further embodiment, the cell is a neuroblastoma cell. In another still further embodiment, the cell is a glioblastoma multiforme cell.

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-42-

In a separate embodiment, the regulatory element is a promoter. In a further embodiment, the promoter is a tissue-specific promoter. In another embodiment, the promoter is an inducible promoter.

5

The linked *mda-7* gene may be introduced into the cells by naked DNA technology, retroviral vectors, antibody-coated liposomes, mechanical or electrical means. These technologies are known in the art.

10

This invention also provides a method of determining the stage of a melanoma comprising: (a) obtaining appropriate amount of cells from the melanoma; (b) measuring the expression level of the *mda-7* gene in the cells; and (b) comparing the expression level with predetermined standards of normal and melanoma cells in different stages, thereby determining the stage of a melanoma.

15

In an embodiment, the expression is measured by the antibodies against the *mda-7* protein. In another embodiment, the expression is measured by in situ hybridization.

20

This invention also provides a method for indicating the effectiveness of a treatment against cancer comprising measuring the expression level of *mda-7* gene in the cells of the cancer, the increase of the expression level indicating the effectiveness of the treatment. The cancer may be a melanoma, leukemia, lymphoma, neuroblastoma or a glioblastoma multiforme tumor.

25

30

This invention provides a method of determining whether a cell is senescent comprising detecting the expression of *mda-7*, the expression of the *mda-7* gene indicating that the cell is senescent.

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-43-

This invention provides a method of identifying a compound inhibiting senescence comprising: a) incubating a plurality of cells with an appropriate amount of a compound; b) detecting the expression of *mda-7*, the inhibition of the expression of *mda-7* indicating that the compound is inhibiting senescence.

This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-8*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

In a separate embodiment, the *mda-8* gene is coding for a human protein.

This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-8*.

This invention provides a method of detecting the expression of *mda-8* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules capable of *mda-8*, under conditions permitting hybrids formation; and c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-8* gene in the cell.

This invention provides a method for distinguishing a glial cell from a malignant astrocytoma cell comprising detecting the expression of *mda-8* gene using the above-

-44-

described method, the expression of *mda-8* gene indicating that the cell is a normal glial cell.

5 The invention also provides a method for monitoring the response of a cell to an anticancer agent such as actinomycin D, adriamycin or cis-platinum comprising detecting the expression of *mda-8* gene using the above-described method, the expression of *mda-8* gene indicating that the cell responds to the anticancer agent.

10 The invention further provides a method for monitoring the response of a cell to DNA damage induced by UV irradiation comprising detecting the expression of *mda-8* gene using the above-described method, the expression of  
15 *mda-8* gene indicating that the cell responds to the DNA damage.

This invention provides a method for detecting type II  
20 interferons in a sample comprising: a) incubating the sample with a target cell containing the 5' regulatory element of *mda-8* permitting binding of type II interferon transcriptional regulatory proteins to the 5' regulatory element; and b) detecting the binding, the binding indicating the presence of type II interferons in the  
25 sample.

In an embodiment, the cell is an eukaryotic cell.

30 In another embodiment, the 5' regulatory element is linked to the native *mda-8* gene and the detection of binding is by the examination of the elevated level of *mda-8* gene expression.

35 In one embodiment, the 5' regulatory element is linked to a marker gene. In a still further embodiment, the marker

-45-

gene is  $\beta$ -galactosidase, luciferase or CAT.

5 This invention provides a method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising: a) inducing appropriate concentration of the human melanoma cells with an appropriate concentration of the compound; and b) detecting the expression of *mda-8* using the above-described method, the expression of *mda-8* gene indicating  
10 that the compound is capable of inducing terminal differentiation in human melanoma cells.

This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation  
15 associated gene designated *mda-9*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

In a separate embodiment, the *mda-9* gene is coding for a  
20 human protein.

This invention provides an isolated nucleic acid molecule of an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene  
25 designated *mda-9*, wherein the protein is a human protein.

This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of an  
30 isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-9*.

This invention provides a method of detecting the  
35 expression of *mda-9* gene in a cell comprising: a)

-46-

isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-9* under conditions permitting hybrids formation; and c) detecting hybrids formed, detection of hybrids formed indicating the expression of *mda-9* gene in the cell.

This invention provides a method for indicating the stage of progression of a human melanoma cell comprising detecting the expression of *mda-9* gene using the method of detecting the expression of *mda-9* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acids with the nucleic acid molecules of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-9* under conditions permitting hybrids formation; and c) detecting hybrids formed, detection of hybrids formed indicating the expression of *mda-9* gene in the cell, the expression of *mda-9* gene indicating the stage of progression of the human melanoma cell.

This invention provides a method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising: a) incubating appropriate concentration of the human melanoma cells with an appropriate concentration of the compound; b) detecting the expression of *mda-9* using the method of detecting the expression of *mda-9* gene in a cell

-47-

comprising: a) isolating the nucleic acids in the cell;  
b) hybridizing the isolated nucleic acids with the  
nucleic acid molecules of at least 15 nucleotides capable  
of specifically hybridizing with a sequence of the  
5 nucleic acid molecule of an isolated nucleic acid  
molecule encoding a protein produced by a melanoma  
differentiation associated gene designated *mda-9* under  
conditions permitting hybrids formation; and c) detecting  
hybrids formed, detection of hybrids formed indicating  
10 the expression of *mda-9* gene in the cell, the expression  
of *mda-9* gene indicating that the compound is capable of  
inducing terminal differentiation in human melanoma  
cells.

15 This invention provides a method for identifying a  
compound capable of inducing specific patterns of DNA  
damage caused by UV irradiation and gamma irradiation in  
human melanoma cells comprising: a) inducing appropriate  
concentration of the human melanoma cells with an  
20 appropriate concentration of the compound; and b)  
detecting the expression of *mda-9* using the method of  
detecting the expression of *mda-9* gene in a cell  
comprising: a) isolating the nucleic acids in the cell;  
b) hybridizing the isolated nucleic acids with the  
25 nucleic acid molecules of at least 15 nucleotides capable  
of specifically hybridizing with a sequence of the  
nucleic acid molecule of an isolated nucleic acid  
molecule encoding a protein produced by a melanoma  
differentiation associated gene designated *mda-9* under  
30 conditions permitting hybrids formation; and c) detecting  
hybrids formed, detection of hybrids formed indicating  
the expression of *mda-9* gene in the cell, the expression  
of *mda-9* gene indicating that the compound is capable of  
inducing specific patterns of DNA damage caused by UV  
35 irradiation and gamma irradiation in human melanoma cells.

-48-

The invention also provides a method for identifying the presence of tumor necrosis factor or a similarly acting agent comprising detecting the expression of *mda-9* gene using the above-described method, the expression of *mda-9* gene indicating that the tumor necrosis factor or similar agent is present.

The invention further provides a method for monitoring the response of a cell to an anticancer agent such as phenyl butyrate or VP-16 comprising detecting the expression of *mda-9* gene using the above-described method, the expression of *mda-9* gene indicating that the cell responds to the anticancer agent.

In a separate embodiment, the *mda-9* gene is coding for a human protein.

This invention provides a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of *mda-9*.

This invention provides a method of detecting the expression of *mda-9* gene in a cell comprising: a) isolating the nucleic acids in the cell; b) hybridizing the isolated nucleic acid with the nucleic acid capable of specifically hybridizing with *mda-9* under conditions permitting hybrid formation and c) detecting hybrids formed, detection of hybrids formed indicating the expression of *mda-9* gene in the cell.

This invention provides a method for distinguishing an early stage and more progressed human melanoma cell comprising detecting the expression of *mda-9* gene indicating that the cell is more progressed human melanoma cell.



-49-

This invention provides a method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising: a) incubating human melanoma cell with the compound effective of inducing terminal differentiation in human melanoma cells; and b) detecting the expression of *mda-9* using the above-described method, the expression of *mda-9* gene indicating that the compound is capable of inducing terminal differentiation in human melanoma cells.

10

This invention provides a method for identifying a compound capable of inducing specific patterns of DNA damage caused by UV irradiation and gamma irradiation in human melanoma cells comprising: a) incubating human melanoma cells with the compound effective of inducing specific patterns of DNA damage caused by UV irradiation and gamma irradiation in human melanoma cells; and b) detecting the expression of *mda-90* using the above-described method, the expression of *mda-9* gene indicating that the compound is capable of inducing specific patterns of DNA damage caused by UV irradiation and gamma irradiation in human melanoma cells.

20

This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-11*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

25

In a separate embodiment, the *mda-11* gene is coding for a human protein.

30

This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-14*. In an embodiment, the

35

-50-

nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

5 In a separate embodiment, the *mda-14* gene is coding for a human protein.

10 This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-17*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

15 In a separate embodiment, the *mda-17* gene is coding for a human protein.

20 This invention provides an isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-18*. In an embodiment, the nucleic acid is a cDNA. In another embodiment, the nucleic acid is genomic DNA.

In a separate embodiment, the *mda-18* gene is coding for a human protein.

25 In an embodiment, the above-described isolated nucleic acid molecule is cDNA. In another embodiment, the nucleic acid molecule is from humans.

30 This invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of protein encoded by the melanoma differentiation associated genes, but which should not produce phenotypic changes. Alternatively, this invention also encompasses DNAs and cDNAs which hybridize to the DNA and cDNA of the  
35 subject invention. Hybridization methods are well known

-51-

to those of skill in the art.

The DNA molecules of the subject invention also include DNA molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transforming and transfecting prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

Moreover, the isolated nucleic acid molecules encoding a protein coded by the melanoma differentiation associated gene are useful for the development of probes to study

-52-

melanoma differentiation. In addition, the isolated nucleic acid molecules encoding a protein coded by the melanoma differentiation associated gene are useful for screening for agents with anticancer activity, agents  
5 which can induce DNA damage and agents which induce cell growth arrest in human melanoma.

The isolated nucleic acid molecules encoding a protein coded by the melanoma differentiation associated gene are  
10 also useful for distinguishing normal from malignant central nervous system cells, adenocarcinomas from carcinomas, and fibroblasts from epithelial cells.

This invention further provides a nucleic acid molecule  
15 of at least 15 nucleotides capable of specifically hybridizing with a sequence of the above-described nucleic acid molecule, i.e., the melanoma differentiation associated genes.

20 This nucleic acid molecule produced can either be DNA or RNA. As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen  
25 bonding between complementary base pairs.

When a situation arises that requires the nucleic acid molecule to be uniquely recognizing a gene, it is well-known in the art to select regions in the sequence which  
30 will distinguish one gene from the other. Simple experiments may be designed to find such unique regions.

This nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of  
35 the above-described nucleic acid molecule can be used as

-53-

a probe. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or  
5 fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule which encodes a protein produced by a melanoma differentiation associated gene into suitable vectors, such as plasmids or bacteriophages, followed by  
10 transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

15 RNA probes may be generated by inserting the above-described isolated nucleic acid molecule downstream of a bacteriophage promoter such as T3, T7 or SP6. Large amounts of RNA probe may be produced by incubating the  
20 labeled nucleotides with the linearized fragment containing the above-described molecule where it contains an upstream promoter in the presence of the appropriate RNA polymerase.

25 This invention also provides a method of detecting expression of a melanoma differentiation associated gene in a cell which comprises obtaining total cellular RNA or mRNA from the cell, contacting the total cellular RNA or mRNA so obtained with a labelled nucleic acid molecule of  
30 at least 15 nucleotides capable of specifically hybridizing with a sequence of the above-described nucleic acid molecule under hybridizing conditions, determining the presence of total cellular RNA or mRNA hybridized to the molecule, and thereby detecting the  
35 expression of the melanoma differentiation associated

-54-

gene in the cell.

The nucleic acid molecules synthesized above may be used to detect expression of melanoma differentiation associated genes by detecting the presence of the  
5 correspondent RNA or mRNA. Total cellular RNA or mRNA from the cell may be isolated by many procedures well known to a person of ordinary skill in the art. The hybridizing conditions of the labelled nucleic acid molecules may be determined by routine experimentation  
10 well known in the art. The presence of total cellular RNA or mRNA hybridized to the probe may be determined by gel electrophoresis or other methods known in the art. By measuring the amount of the hybrid made, the expression of the melanoma differentiation associated  
15 genes by the cell can be determined. The labelling may be radioactive. For an example, one or more radioactive nucleotides can be incorporated in the nucleic acid when it is made.

20 In one embodiment of this invention, nucleic acids are extracted from lysed cells and the mRNA is isolated from the extract using an oligo-dT column which binds the poly-A tails of the mRNA molecules. The mRNA is then exposed to radioactively labelled probe on a  
25 nitrocellulose membrane, and the probe hybridizes to and thereby labels complementary mRNA sequences. Binding may be detected by luminescence autoradiography or scintillation counting. However, other methods for performing these steps are well known to those skilled in  
30 the art, and the discussion above is merely an example.

This invention also provides a method of detecting expression of a melanoma differentiation associated gene in tissue sections which comprises contacting the tissue  
35 sections with a labelled nucleic acid molecule of at

-55-

least 15 nucleotides capable of specifically hybridizing with a sequence of the above-described nucleic acid molecule under hybridizing conditions, determining the presence of mRNA hybridized to the molecule, and thereby  
5 detecting the expression of the melanoma differentiation associated gene in tissue sections.

This invention also provides the above-describe nucleic acid molecules operatively linked to a promoter of RNA  
10 transcription.

This invention provides vectors which comprise the above-described isolated nucleic acid molecules. In an embodiment, the vector is a plasmid.

15 Various vectors including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses are well known to ordinary skilled practitioners. This invention further provides a vector which comprises the isolated  
20 nucleic acid molecule encoding a protein produced by a melanoma differentiation gene.

As an example to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create  
25 complementary ends on both molecules which base pair with each other and are then ligated together with DNA ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which  
30 cuts at that site. Other means are also available and known to an ordinary skilled practitioner.

In an embodiment, the nucleic acid molecule is cloned in the XhoI/EcoRI site of pBlueScript. Plasmids, *mda-1*,  
35 *mda-4*, *mda-5*, *mda-6*, *mda-7*, *mda-8*, *mda-9*, *mda-11*, *mda-14*,

-56-

mda-17, and mda-18 were deposited on October 26, 1993 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmids, mda-1, mda-4, mda-5, mda-6, mda-7, mda-8, mda-9, mda-11, mda-14, mda-17, and mda-18 were accorded ATCC Accession Numbers 75582, 75583, 75584, 75585, 75586, 75587, 75588, 75589, 75590, 75591 and 75592 respectively.

In another embodiment, a 3' fragment of the mda-6 gene is cloned in the EcoRI and XbaI site of the pBluescript plasmid and designed as mda-6.3'. The mda-6.3' was deposited on September 30, 1994 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, mda-6.3' was accorded ATCC Accession Numbers 75903.

In another embodiment, a 5' fragment of the mda-6 gene is cloned in the salI site of the pSP64 plasmid and designed as mda-6.5'. The mda-6.5' was deposited on September 30, 1994 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, mda-6.5' was accorded ATCC Accession Number 75904.

Plasmids mda-6.3' and mda-6.5' constitute the full-length of the mda-6 gene. An ordinary skilled artisan can



-57-

easily obtain the inserts from the plasmids and ligate the inserts to obtain the full-length gene.

5 In another embodiment, a 3' fragment of the *mda-7* gene is cloned in the EcoRI and XbaI site of the pBluescript plasmid and designed as *mda-7.3'*. The *mda-7.3'* was deposited on September 30, 1994 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of  
10 the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, *mda-7.3'* was accorded ATCC Accession Number 75905.

15 In another embodiment, a 5' fragment of the *mda-7* gene is cloned in the salI site of the pSP64 plasmid and designed as *mda-7.5'*. The *mda-7.5'* was deposited on September 30, 1994 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.  
20 under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid, *mda-7.5'* was accorded ATCC Accession Numbers 75906.

25 Plasmids *mda-7.3'* and *mda-7.5'* constitute the full-length of the *mda-7* gene. An ordinary skilled artisan can easily obtain the inserts from the plasmids and ligate the inserts to obtain the full-length gene.

30 This invention provides a host vector system for the production of a polypeptide having the biological activity of a protein encoded by melanoma differentiation associated gene which comprises the above-described vector and a suitable host. These vectors may be  
35 transformed into a suitable host cell to form a host cell

-58-

vector system for the production of a protein produced by the melanoma differentiation associated genes.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general. Expression vectors are useful to produce cells that express the protein produced by the melanoma differentiation associated genes.

This invention further provides an isolated DNA or cDNA molecule described hereinabove wherein the host cell is selected from the group consisting of bacterial cells (such as E.coli), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

As stated above and in the text which follows, this invention provides gene(s) which express when a cell becomes terminally differentiated and irreversibly growth arrested, treated with a DNA damaging agent and/or exposed to an anticancer agent. Therefore, this invention is useful for inducing a target cell to a

-59-

terminally differentiated stage. Such a target cell may be a cancerous cell such as a melanoma cell or a glioblastoma multiforme cell. The gene which expresses when a cell becomes terminally differentiated may be introduced into the target cell via retroviral technology or other technologies known in the art. The gene may be controlled by its own promoter or other heterologous promoters. Expression of this gene will then result in terminal differentiation and an irreversible loss of proliferative capacity in the cancerous cell.

This invention has also provided nucleic acid molecules which will suppress the terminal differentiation of a cell. Such molecules are useful for preventing terminal differentiation by switching off a target gene in a cell which has been treated with differentiation inducing agents, anticancer agents or DNA damaging agents. The target gene may be switched off via antisense technology. After the gene has been switched off, normal cells, such as bone marrow stem cells, can be prevented from becoming terminally differentiated and irreversibly growth arrested when treated with differentiation inducing agents, anticancer agents or DNA damaging agents.

Antisense technology is well known in the art. Essentially, a segment of the melanoma differentiation associated gene will be selected to be the antisense sequence. The expression of the antisense sequence will switch off the expression of the gene. The antisense sequence may be introduced into the cell via technologies which are well known in the art, such as electroporation transduction, retroviral insertion or liposome-mediated gene transfer.

This invention also provides a method of producing a

-60-

polypeptide having the biological activity of a protein encoded by melanoma differentiation associated gene which comprises growing the host cells of the above-described host vector system under suitable conditions permitting  
5 production of the polypeptide and recovering the polypeptide so produced.

This invention provides a mammalian cell comprising a DNA molecule encoding a protein produced by a melanoma  
10 differentiation associated gene, such as a mammalian cell comprising a plasmid adapted for expression in a mammalian cell, which comprises a DNA molecule encoding a protein produced by the melanoma differentiation associated gene and the regulatory elements necessary for  
15 expression of the DNA in the mammalian cell so located relative to the DNA encoding a protein produced by a melanoma differentiation gene as to permit expression thereof.

20 Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH3T3, CREF cells, CHO cells, HeLa cells, Ltk<sup>+</sup> cells, Cos cells, etc. Expression plasmids such as that described supra may be used to transfect mammalian cells by methods well  
25 known in the art such as calcium phosphate precipitation, electroporation or the plasmid may be otherwise introduced into mammalian cells, e.g., by microinjection, to obtain mammalian cells which comprise DNA, e.g., cDNA or a plasmid, encoding a protein produced by a melanoma  
30 differentiation associated gene.

Also provided by this invention is a purified protein encoded by the above-described isolated nucleic acid molecule. As used herein, the term "purified protein"

-61-

shall mean isolated naturally-occurring protein encoded by a melanoma differentiation associated gene (purified from nature or manufactured such that the primary, secondary and tertiary conformation, and posttranslational modifications are identical to naturally-occurring material) as well as non-naturally occurring polypeptides having a primary structural conformation (i.e. continuous sequence of amino acid residues). Such polypeptides include derivatives and analogs.

This invention also provides a method to produce antibody using the above purified protein. In an embodiment, the antibody is monoclonal. In another embodiment, the antibody is polyclonal.

This invention further provides antibodies capable of binding to the purified protein produced by the melanoma differentiation associated genes.

With the protein sequence information which can either be derived from the above described nucleic molecule or by direct protein sequencing of the above described purified protein, antigenic areas may be identified and antibodies directed against these areas may be generated and targeted to the cancer for imaging the cancer or therapies.

This invention provides a method to select specific regions on the protein produced by a melanoma differentiation associated gene to generate antibodies. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build. In the case of cell membrane.

-62-

proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer of the cell membrane, while hydrophilic regions are located on the cell surface, in an aqueous environment. Usually, the hydrophilic regions will be more immunogenic than the hydrophobic regions. Therefore the hydrophilic amino acid sequences may be selected and used to generate antibodies specific to protein produced by the melanoma differentiation genes.

10 The selected peptides may be prepared using commercially available machines. As an alternative, DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen.

15 Polyclonal antibodies against these peptides may be produced by immunizing animals using the selected peptides. Monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing B cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Alternatively, monoclonal antibodies may be produced by in vitro techniques known to a person of ordinary skill in the art. These antibodies are useful to detect the expression of protein produced by the melanoma differentiation associated genes in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

25

30 This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

35

-63-

## Experimental Details

### First Series of Experiments

#### 5 MATERIALS AND METHODS

##### Cell Line, Growth Conditions, and Preparation of Conditioned Medium

10 The H0-1 cell line is a melanotic melanoma derived from  
a 49-year-old female and was used between passage 100 and  
125 (6,8,13). H0-1 cells were kindly provided by Dr.  
Beppino C. Giovanella, Stehlin Foundation for Cancer  
Research, Houston, Texas. Cultures were grown at 37 °C  
15 in a 95% air 5% CO<sub>2</sub>-humidified incubator in Dulbecco's  
modified Eagle's medium (DMEM) supplemented with 10%  
fetal bovine serum (DMEM-10) (Hyclone, Logan, UT). H0-1  
cells were maintained in the logarithmic stage of growth  
by subculturing (1:5 or 1:10) prior to confluency  
20 approximately every 4 to 5 days. The effect of IFN- $\beta$   
(2000 units/ml), IFN- $\gamma$  (2000 units/ml), IFN- $\beta$  + IFN- $\gamma$   
(1000 units/ml of each interferon), MPA (3.0  $\mu$ M), RA (2.5  
 $\mu$ M), MEZ (10 ng/ml), IFN- $\beta$  + MEZ (2000 units/ml + 10  
ng/ml), MPA + MEZ (3.0  $\mu$ M + 10 ng/ml, and RA + MEZ (2.5  
25  $\mu$ M + 10 ng/ml) on growth was determined after 4 days of  
treatment, as described previously (6,8,9). Terminal  
cell differentiation, with a concomitant loss of  
proliferative capacity, was assayed by treating cells  
with the various agents for either 4 or 7 days, washing  
30 cultures two times with serum-free DMEM, followed by  
incubation in DMEM-10 in the absence of inducer(s) for an  
additional 14 days. Total cell numbers were determined  
after days 4, 7, 14, and 21, using a Z<sub>M</sub> Coulter Counter,  
and viable cell numbers were determined by trypan blue

-64-

dye exclusion (6). Terminal cell differentiation was indicated if no proliferation occurred, but cells remained viable, after growth for 14 days, with medium changes every 3 or 4 days, in the absence of the inducer(s). Increases in cell number (two or more population doublings) after removal of the inducing agent(s) was considered reversible growth suppression. Conditioned medium was prepared from H0-1 cells treated with a high dose of MEZ (50 ng/ml), IFN- $\beta$  + MEZ (2000 U/ml + 10 ng/ml), MPA + MEZ (3.0  $\mu$ M + 10 ng/ml), or RA + MEZ (2.5  $\mu$  + 10 ng/ml) for 24 hours followed by three washes in DMEM without FBS and growth for 72 hours in inducer-free medium (DMEM-10). Conditioned medium was collected from treated cultures, contaminating cells were removed by centrifugation at 1000 rpm/10 min., and conditioned medium was stored at 4°C until assayed for gene modulatory activity. Control conditioned medium was obtained as experimental conditioned medium from cells receiving only a medium change 24 hours after plating in the absence of test compound(s) and growth for 72 hours in DMEM-10.

#### RNA Isolation and Northern Hybridization Analysis

Steady-state levels of specific mRNAs were determined by Northern blotting analysis of total RNA probed with appropriate  $^{32}$ P-labeled gene probes as described previously (14-16). RNAs were analyzed from cells treated with inducer for 24 hours, treated for 24 hours with inducer followed by growth for 72 hours in the absence of inducer, or treated continuously for 96 hours with the inducer. The concentration of inducing compounds used were the same as those used for growth studies. The effect of conditioned medium on gene



-65-

expression changes was determined by treating H0-1 cells for 24 hours with a 1:2 dilution of conditioned medium (equal volumes of conditioned medium and DMEM supplemented with 10% fetal bovine serum (DMEM-10) or for 5 96 hours with a 1:4 dilution of conditioned medium (1 vol. of conditioned medium to 3 vol. of DMEM-10). The probes used in the present study were specific for  $\beta$ -actin (17),  $\gamma$ -actin (17), c-jun (18), c-myc (19), fibronectin (20), gro/MGSA (21), HLA Class I antigen 10 (22), HLA Class II antigen (HLA-DR<sub>β</sub>) (22),  $\alpha_5$  integrin (23),  $\beta_1$  integrin (24), ISG-15 (25), ISG-54 (25), jun-B (26), and tenascin (27). Northern blots were also probed with a <sup>32</sup>P-labeled GAPDH gene (15) to verify similar mRNA expression under the various experimental conditions. 15 Following hybridization, the filters were washed and exposed for autoradiography.

#### Reagents

20 Recombinant human IFN- $\beta$ , with a serine substituted for a cysteine at position 17 of the molecule (28), was kindly provided by Triton Bioscience (Alameda, CA). IFN- $\beta$  was obtained as a lyophilized powder with a concentration of  $4.5 \times 10^7$  U/ml. Recombinant human immune interferon (IFN- 25  $\gamma$ ) was produced and purified to homogeneity as described previously (29). IFN- $\gamma$  was kindly provided by Dr. Sidney Pestka, UMDNJ-Robert Wood Johnson Medical School, Piscataway, New Jersey. The interferon titers were determined using a cytopathic effect inhibition assay 30 with vesicular stomatitis virus on a bovine kidney cell line (MDBK) or human fibroblast AG-1732 cells (30). Concentrated stocks of IFN- $\beta$  and IFN- $\gamma$  were diluted to  $1 \times 10^6$  U/ml in DMEM-10, frozen at -80°C, thawed immediately prior to use, and diluted to the appropriate

-66-

concentration in DMEM-10. MEZ, RA, and MPA were obtained from Sigma Scientific Co. (St. Louis, MO). Stock solutions were prepared in dimethyl sulfoxide (DMSO), aliquoted into small portions, and stored at -20°C. The  
5 final concentration of DMSO used in solvent controls was 0.01%. This concentration of DMSO did not alter growth, melanin synthesis, tyrosinase activity, or antigen expression in HO-1 cells.

## 10 EXPERIMENTAL RESULTS

### Induction of Reversible and Irreversible Growth Suppression (Terminal Cell Differentiation) in HO-1 Cells

15 The effect of RA, MPA, MEZ, IFN- $\beta$  and IFN- $\gamma$ , used alone or in various combinations, on growth (reversible and irreversible growth suppression), melanin synthesis, tyrosinase activity, and cellular morphology of HO-1  
20 cells is summarized in Table 1. The most effective agents in inhibiting HO-1 growth were the combinations of IFN- $\beta$  + MEZ and IFN- $\beta$  + IFN- $\gamma$  (Figure 1). The relative order of antiproliferative activity of the remaining compounds was MPA = MPA + MEZ > IFN- $\gamma$  > MEZ = RA + MEZ. In the case of RA, no inhibition of growth occurred.  
25 Treatment of HO-1 cells with IFN- $\beta$  + MEZ for 96 hours resulted in an irreversible loss of proliferative capacity, that is, terminal cell differentiation. This was indicated by the failure of treated cells to resume growth, even though they remained viable, after removal  
30 of the test agents. In contrast, all of the other compounds resulted in a reversible inhibition of growth (data not shown). These findings indicate a dissociation between growth suppression and terminal cell differentiation in HO-1 cells.

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-67-

Treatment of H0-1 cells with MPA, MEZ, RA + MEZ, MPA + MEZ, or IFN- $\beta$  + MEZ resulted in distinctive morphologic changes that were characterized by dendrite-like processes (data not shown) (6,8,10). RA, MPA, MEZ, IFN- $\beta$ , and IFN- $\beta$  + MEZ have been shown previously to enhance melanin synthesis, a marker of melanoma differentiation, in H0-1 cells (6,8,12). In contrast, IFN- $\gamma$ , alone or in combination with IFN- $\beta$ , did not induce morphologic changes or increase melanin levels above

TABLE 1

	Experimental Conditions <sup>a</sup>	Morphology changes <sup>b</sup>	Melanin Synthesis <sup>c</sup>	Tyrosinase activity <sup>d</sup>
15	RA (2.5 $\mu$ M)	-	1+	2+
	MPA (3.0 $\mu$ M)	+	2+	3+
20	MEZ (10 ng/ml)	+	1+	NT
	IFN- $\beta$ (2000 U/ml)	-	1+	NT
25	IFN- $\gamma$ (2000 U/ml)	-	-	NT
	RA + MEZ (2.5 $\mu$ M + 10 ng/ml)	+	NT	NT
30	MPA + MEZ (3.0 $\mu$ M + 10 ng/ml)	+	NT	NT
35	IFN- $\beta$ + IFN- $\gamma$ (1000 U/ml + 1000 U/ml)	-	1+	NT
40	IFN- $\beta$ + MEZ (2000 U/ml + 10 ng/ml)	+	4+	NT
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-68-

TABLE 1 (Continued)

	Experimental conditions <sup>a</sup>	Growth Suppression (reversible) <sup>c</sup>	Terminal cell differentiation <sup>f</sup>
5	RA (2.5 $\mu$ M)	-	-
	MPA (3.0 $\mu$ M)	3+	-
10	MEZ (10 ng/ml)	1+	-
	IFN- $\beta$ (2000 U/ml)	3+	-
15	IFN- $\gamma$ (2000 U/ml)	2+	-
20	RA + MEZ (2.5 $\mu$ M + 10 ng/ml)	1+	-
	MPA + MEZ (3.0 $\mu$ M + 10 ng/ml)	3+	-
25	IFN- $\beta$ + IFN- $\gamma$ (1000 U/ml + 1000 U/ml)	4+	-
30	IFN- $\beta$ + MEZ (2000 U/ml + 10 ng/ml)	4+ <sup>e</sup>	+
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40	H0-1 cells were grown for 96 hr or for 6 or 7 days (with medium changes after 3 or 4 days) in the presence of the agents indicated. For morphology, cells grown for 96 hr in the test agent were observed microscopically. For melanin synthesis, results are for 6-d assays for RA and MPA (11) or 7-d assays for MEZ, IFN- $\beta$ , IFN- $\gamma$ , IFN- $\beta$ + IFN- $\gamma$ and IFN- $\beta$ + MEZ (6,12). For tyrosinase assays, results are for 6-d assays for RA, MPA and MEZ (10). Growth suppression (reversible and terminal cell differentiation) assays, refer to cultures treated with the indicated compound(s) for 96 hr prior to cell number determination, or treated for 96 hr and then grown for 2 weeks (with medium changes every 4 days) in the absence of compound prior to cell		
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-69-

number determination.

5       <sup>b</sup> Morphology changes refer to the development of dendrite-like processes 96 hr after growth in the indicated compound. + = presence of dendrite-like processes; - = no dendrite-like processes.

10       <sup>c</sup> Melanin assays were determined as described in refs. 6,8,11,12. Results are expressed as relative increases based on separate data presented in refs. 6,8,11,12. N.T. = not tested.

15       <sup>d</sup> Tyrosinase assays were performed as described in ref. 11. Relative increases (of a similar magnitude) were found for RA, MPA and MEZ after 6 days exposure to these agents (11). N.T. = not tested.

20       <sup>e</sup> Reversible growth suppression indicates resumption of cell growth after treatment with the indicated compound(s) for 96 hr, removal of the test agent and growth for 14 days in compound(s) free medium. Further details can be found in materials and methods. The degree of initial 96 hr growth suppression is indicated as: - = no significant change in growth (< 10% reduction in growth in comparison with untreated control cultures); 1+ = ~30% reduction in growth in comparison with untreated control cultures; 2+ = ~40% reduction in growth in comparison with untreated control cultures; 3+ = ~50 to 60% reduction in growth in comparison with untreated control cultures; 4+ = ~80% reduction in growth in comparison with untreated control cultures.

35       <sup>f</sup> The combination of IFN- $\beta$  + MEZ results in irreversible growth suppression.

40       <sup>g</sup> The combination of IFN- $\beta$  + MEZ results in irreversible growth suppression

45       that induced by IFN- $\beta$  alone (10,12). With the exception of IFN- $\beta$  + MEZ, morphologic and melanin changes were reversible following removal of the test agent(s) (data not shown). These data indicate that specific cellular and biochemical changes induced in H0-1 cells, such as reversible growth suppression, melanin synthesis, and morphologic changes, can occur with or without the

-70-

induction of terminal cell differentiation. However, an irreversible loss of proliferative capacity with the retention of cell viability is a property unique to the terminal cell differentiation phenotype.

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Changes in the Expression of Early Growth Response and Interferon-Responsive Genes During Reversible and Irreversible Growth Suppression (Terminal Cell Differentiation) in H0-1 Cells

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Initial studies were conducted to determine the effect of the various differentiation and growth modulating agents on the 96-hour expression of the early response genes *c-fos*, *c-jun*, *jun-B*, *jun-D*, and *c-myc* (Figure 2). None of the experimental treatments resulted in altered *c-fos* expression and no hybridization was obtained with RNA isolated from control or treated cells probed with *jun-D* (data not shown). Increases were observed, however, in both *c-jun* and *jun-B* expression in H0-1 cells treated for 96 hours with all of the test agents, with the exception of IFN- $\beta$  and RA (see Figure 2). The magnitude of the increase was similar in H0-1 cells treated with IFN- $\gamma$ , MEZ, or MPA and was greatest for H0-1 cells treated with IFN- $\beta$  + MEZ, MPA + MEZ, or RA + MEZ (see Figure 2). Unlike *c-jun* and *jun-B* expression *c-myc* expression was downregulated in H0-1 cells grown for 96 hours in MEZ, MPA, IFN- $\beta$  + MEZ, MPA + MEZ, and RA + MEZ (Figure 2). The magnitude of suppression was greater in H0-1 cells treated with IFN- $\beta$  + MEZ, MPA + MEZ, and RA + MEZ. In contrast, treatment of H0-1 cells with IFN- $\gamma$ , alone or in combination with IFN- $\beta$ , resulted in increased *c-myc* expression.

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To determine the temporal relationship between treatment with IFN- $\beta$  + MEZ, MPA + MEZ, and RA + MEZ and changes in

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-71-

c-jun, jun-B, and c-myc levels, HO-1 cells were treated with the inducers for 24 h and total cytoplasmic RNA was isolated and analyzed by Northern blotting (Figure 3). Because many of the effects observed when MEZ is combined with IFN- $\beta$ , MPA, or RA are observed to a lesser extent in cells treated with 10 mg/ml of MEZ alone, RNA was also isolated from HO-1 cell treated for 24 h with a high dose of MEZ (50 ng/ml) (Figure 3). Under these experimental conditions, c-jun and jun-B expression were induced under all experimental conditions and c-myc expression was only marginally reduced in cultures treated with IFN- $\beta$  + MEZ, MPA + MEZ, and RA + MEZ. Twenty-four-hour exposure to IFN- $\beta$  + MEZ resulted in the largest induction in c-jun and jun-B expression.

To determine if the gene expression changes induced during the induction of reversible and irreversible growth suppression/differentiation persist in HO-1 cells treated with specific inducers, cultures were grown for 24 h in the presence of inducer and then incubated for an additional 72 h in inducer-free medium prior to isolating total cellular RNA (Figure 3). Under these experimental conditions, c-jun and jun-B expression were induced to the greatest extent in IFN- $\beta$  + MEZ-treated cultures. Smaller increases in c-jun and jun-B expression were apparent in high-dose MEZ-, MPA + MEZ-, and RA + MEZ-treated HO-1 cells. In the case of c-myc, expression was dramatically reduced in IFN- $\beta$  + MEZ-treated cultures and reduced to a lesser extent in MPA + MEZ- and RA + MEZ-treated cultures. These results suggest that the hierarchy for inducing c-jun, jun-B, and c-myc gene expression changes in HO-1 cells is IFN- $\beta$  + MEZ > MPA + MEZ > RA + MEZ. As will be discussed, this same pattern of potency in inducing gene expression changes in HO-1 cells is also observed with a number of additional genes.

-72-

Treatment of HO-1 cells for 96 h with the combination of IFN- $\beta$  + MEZ, MPA + MEZ, or RA + MEZ resulted in the enhanced expression of the cytokine-responsive genes HLA Class I antigen and gro/MGSA (Figure 2). In contrast, treatment with IFN- $\beta$ , MEZ, MPA, or RA alone did not significantly alter HLA Class I antigen gene expression or induce gro/MGSA gene expression. Treatment of HO-1 cells for 96 h with IFN- $\gamma$ , alone or in combination with IFN- $\beta$ , also enhanced HLA Class I antigen expression in HO-1 cells, whereas it did not induce gro/MGSA expression (Figure 2). In contrast, although a 96-h exposure of HO-1 cells to IFN- $\gamma$ , alone and in combination with IFN- $\beta$ , enhanced expression of the HLA Class II antigen gene (HLA-DR $_{\beta}$ ), expression of this gene was not significantly enhanced by treatment with IFN- $\beta$  + MEZ, MPA + MEZ, or RA + MEZ (Figure 2). These observations indicate possible autocrine loops involving both a type I interferon (leukocyte interferon IFN- $\alpha$ ) and IFN- $\beta$  as opposed to a type II interferon (IFN- $\gamma$ ) and gro/MGSA in the induction of gene expression changes occurring during both reversible (MPA + MEZ and RA + MEZ) and terminal cell differentiation (IFN- $\beta$  + MEZ).

To determine if an interferon or an interferon-like molecule might be associated with the induction of reversible or irreversible differentiation or both in HO-1 cells, the effect of the various differentiation-inducing and growth-suppressing agents on expression of the interferon-responsive genes, ISG-15 and ISG-54 (25, 31, 32) were determined. As can be seen in Figure 2, treatment of HO-1 cells for 96 h with the combination of IFN- $\beta$  + MEZ, MPA + MEZ, or RA + MEZ resulted in the induction in ISG-15 and ISG-54 gene expression.



- 73 -

Further support for a type I interferon and a gro/MGSA autocrine loop in the reversible commitment to differentiation (MPA + MEZ) and terminal cell differentiation (IFN- $\beta$  + MEZ) processes in HO-1 cells was indicated by analysis of gene expression changes occurring in cultures treated with inducers for 24 h or in cultures treated with inducers for 24 h followed by growth in the absence of inducers for 72 h (Figure 3). Growth of HO-1 cells for 24 h in the presence of IFN- $\beta$  + MEZ resulted in the induction or enhanced expression of the gro/MGSA, HLA Class I antigen, and ISG-15 gene. Similarly, a 24-h treatment with MPA + MEZ induced expression of gro/MGSA to a similar extent as IFN- $\beta$  + MEZ, whereas the effects on HLA Class I antigen and ISG-15 expression were more modest. In contrast, a 24-h treatment with RA + MEZ or a high dose of MEZ did not induce gro/ MGSA or ISG-15 expression, but these treatments did induce a modest increase in HLA Class I antigen expression (Figure 3). Treatment of HO-1 cells for 24 h with inducer (IFN- $\beta$  + MEZ, MPA + MEZ, RA + MEZ or a high dose of MEZ) followed by growth for 72 hours in the absence of inducer resulted in the following changes in HO-1 gene expression: (1) gro/MGSA was induced only by IFN- $\beta$  + MEZ treatment; (2) enhanced HLA Class I antigen expression was induced by all of the treatments with the following potencies, IFN- $\beta$  + MEZ > MPA + MEZ  $\approx$  high dose MEZ > RA + MEZ; and (3) ISG-15 was induced to a similar extent by IFN- $\beta$  + MEZ and MPA + MEZ, whereas RA + MEZ and high-dose MEZ did not induce ISG-15 expression.

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Changes in the Expression of Extracellular and Extracellular Matrix Receptor Genes During Reversible and Irreversible Growth Suppression (Terminal Cell Differentiation) in HO-1 cell.

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-74-

Terminal differentiation in HO-1 cells induced by IFN- $\beta$  + MEZ is associated with morphologic changes resulting in the formation of dendrite-like processes and specific biochemical changes, that is, enhanced tyrosinase activity and melanin synthesis (6). Similar morphologic and biochemical changes are induced in HO-1 cells by MEZ (6, 8) and MPA (11). Studies were conducted to determine the possible relationship between these morphologic and biochemical changes and the expression of genes encoding extracellular matrix molecules (fibronectin and tenascin), receptors for extracellular matrix proteins ( $\alpha$ , integrin and  $\beta$ , integrin), and cytoskeleton proteins ( $\beta$ -actin and  $\gamma$ -actin). When treated for 96 h, fibronectin expression was increased under all treatment protocols, with IFN- $\beta$  and RA being the least effective agents in inducing enhanced fibronectin mRNA in HO-1 cells (i.e. signals were only detected after long exposures of Northern blots) (Figure 4 and data not shown). The most effective single agents resulting in enhanced fibronectin expression were IFN- $\gamma$  and MPA, whereas MEZ was less potent in inducing enhanced fibronectin expression (Figure 4). Large increases in fibronectin expression were also observed in HO-1 cells grown for 96 h in the combination of IFN- $\beta$  + IFN- $\gamma$ , IFN- $\beta$  + MEZ, MPA + MEZ, and RA + MEZ. IFN- $\beta$  + MEZ was more effective than MPA + MEZ, RA + MEZ, or a high dose of MEZ in inducing enhanced fibronectin expression after 24-h treatment (Figure 5). IFN- $\beta$  + MEZ also enhanced fibronectin expression to a greater extent than MPA + MEZ, RA + MEZ, and a high dose of MEZ after removing this combination of inducers and growth for 72 h in medium devoid of the inducing agents (Figure 5).

Expression of the tenascin gene in HO-1 cells treated

-75-

with the various differentiation-inducing and growth-suppressing agents was more complex than fibronectin (Figures 4 and 5). Growth of HO-1 cells for 96 h in the presence of IFN- $\beta$ , MEZ, RA, IFN- $\beta$  + MEZ, or RA + MEZ  
5 resulted in decreased tenascin expression whereas IFN- $\gamma$ , MPA, and IFN- $\beta$  + IFN- $\alpha$  resulted in enhanced tenascin expression (Figure 4). Ninety-six-hour treatment with IFN- $\gamma$ , alone or in combination with IFN- $\beta$ , resulted in the greatest increase in tenascin expression. In  
10 contrast, after a 24-h treatment, tenascin expression was not significantly altered by a high dose of MEZ or RA + MEZ, whereas a small increase in tenascin expression was found in IFN- $\beta$  + MEZ- and MPA + MEZ-treated cultures (Figure 5). When cultures were treated for 24 h with  
15 inducer and then grown for 72 h in the absence of inducer, decreased expression of tenascin was observed in high-dose MEZ- and RA + MEZ-treated cultures, whereas only small reductions in tenascin expression were apparent in MPA + MEZ- or IFN- $\beta$  + MEZ-treated HO-1 cells  
20 (Figure 5).

Changes were also observed in the expression of matrix receptor genes for extracellular matrix proteins,  $\alpha_5$ -integrin,  $\beta_1$ -integrin in HO-1 cells grown for (1) 24 h in  
25 the presence of the inducer(s), (2) 24 h in inducer(s) followed by 72 h in the absence of inducer(s), or (3) continuously in inducer(s) for 96 h (Figures 4 and 5). Increases were observed in  $\alpha_5$  integrin expression in HO-1 cells treated for 96 h with IFN- $\beta$  + IFN- $\gamma$ , IFN- $\beta$  + MEZ,  
30 MPA + MEZ and, to a lesser extent, with RA + MEZ (Figure 4). Increased  $\alpha_5$  integrin expression was also apparent in HO-1 cells treated continuously for 24 hrs or treated for 24 hrs followed by 72 hr growth in the absence of inducer(s) to a high dose of MEZ, MPA + MEZ, RA + MEZ, or

-76-

IFN- $\beta$  + MEZ. In contrast,  $\alpha$ , integrin expressed was reduced in cultures treated with RA for 96 h. In the case of the  $\beta$ , integrin, upregulation after 96-h treatment was apparent in cells treated with IFN- $\gamma$ , IFN- $\beta$  + MEZ, MPA + MEZ, and RA + MEZ (Figure 4). The most effective inducer of both  $\alpha$ , and  $\beta$ , integrin expression in HO-1 cells was IFN- $\beta$  + MEZ (Figures 4 and 5). The level of upregulation was greater for  $\alpha$ , integrin than the  $\beta$ , integrin (Figures 4 and 5).

The effect of the various growth-suppressing and differentiation-modulating compounds on expression of cytoskeletal genes ( $\beta$ -actin and  $\gamma$ -actin) in HO-1 cells is shown in Figures 4 and 5. Under most experimental conditions, only small changes were observed in  $\beta$ -actin and  $\gamma$ -actin mRNA levels. In the case of 96-h-treated cultures, both  $\beta$ -actin and, to a greater extent,  $\gamma$ -actin expression were decreased by treatment with a number of agents, resulting in growth suppression. In contrast, RA, which is not growth suppressive in HO-1 cells, did not significantly alter the expression of these cytoskeletal genes. A common change that was generally most pronounced under all three experimental protocols in HO-1 cells, that is, 24-h treatment, 24-h treatment followed by 72-h growth in the absence of inducer, or continuous treatment for 96 h, was the reduction in  $\beta$ -actin and  $\gamma$ -actin expression by IFN- $\beta$  + MEZ.

#### Modulation of Gene Expression in HO-1 Cells by Conditioned Medium Obtained from Differentiation-Inducer-Treated HO-1 Cells.

The studies described previously demonstrated that interferon-responsive genes and the gro/MGSA gene were

-77-

activated during the process of reversible and irreversible differentiation in HO-1 cells. They further suggested the possibility of an involvement of autocrine-feedback pathways in the differentiation process (Figures 2 and 3). To determine directly if HO-1 cells treated with agents inducing a reversible commitment to differentiation (a high dose of MEZ, RA + MEZ, and MPA + MEZ) and/or terminal cell differentiation (IFN- $\beta$  + MEZ) secrete factor(s) that can modulate gene expression in HO-1 cells, conditioned medium was collected from cells treated for 24 h with the inducer followed by growth for 72 h in the absence of inducer (Figures 6 and 7). HO-1 cells were grown in an equal volume of conditioned medium plus and equal volume of DMEM-10 (1:2) for 24 h or with 1 vol of conditioned medium plus 3 vol of DMEM-10 (1:4) for 96 h. Total cytoplasmic RNA was then isolated and analyzed by Northern blotting for the expression of a series of early growth response, interferon responsive, extracellular matrix, extracellular matrix receptor, and cytoskeletal genes (Figures 6 and 7). With the exception of fibronectin and small increases in  $\beta_1$  integrin expression, treatment for 24 h with 1:2 conditioned medium obtained from the other experimental conditions (which result in a reversible commitment to differentiation) did not alter or induce expression of the genes tested, including c-jun, jun-B, c-myc, gro/MGSA, HLA Class I antigen, ISG-15,  $\alpha$ , integrin,  $\beta$ -actin,  $\gamma$ -actin, or tenascin. Exposure of HO-1 cells for 96 h to 1:4 conditioned medium obtained from IFN- $\beta$  + MEZ-treated HO-1 cells also enhanced fibronectin, HLA Class I antigen,  $\beta_1$  integrin and tenascin expression, as well as inducing ISG-15 expression. With the exception of fibronectin and tenascin, which were enhanced by 1:4 conditioned medium obtained from 24-h treated IFN- $\beta$  +

-78-

MEZ, MPA + MEZ, RA + MEZ treated cultures, no modification in the expression of the various genes was apparent using 1:4 conditioned medium from any of the experimental procedures.

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The HO-1 human melanoma cell line can be chemically induced to reversibly express specific markers of differentiation or to undergo terminal cell differentiation. The present study was undertaken to determine which specific programs of gene expression are modified as a consequence of these cellular alterations. Inductions of terminal differentiation, and, to a lesser extent, reversible differentiation, was associated with changes in the expression specific immediate early response, interferon-responsive, cytokine-responsive, extracellular matrix, and extracellular matrix receptor genes. In addition, conditioned medium obtained from HO-1 cells treated with IFN- $\beta$  + MEZ also resulted in similar changes in gene expression in naive HO-1 cells as those observed following direct exposure to the chemical inducers of terminal differentiation. These results indicate that common gene-expression changes are associated with both the reversible and irreversible induction of differentiation in HO-1 cells. In addition, the terminal differentiation process is correlated with the activation of several autocrine pathways involving both IFN- $\beta$  and gro/MGSA.

Immediate early response genes, such as *c-myc*, *c-fos*, *c-jun*, *jun-B*, and *jun-D*, have been shown to be involved in the regulation of growth and/or differentiation in other model systems [33-35]. In the case of *c-myc*, a reduction in expression of this gene is observed in many cell types either induced to terminally differentiate or under conditions resulting in a reduction in cellular growth

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- 79 -

without the induction of differentiation-related genes (36-40). A direct role of *c-myc* expression in regulating differentiation in a variety of model cell culture systems has also been demonstrated using *c-myc* antisense constructs or oligomers (41-45). In specific systems, the downregulation of *c-myc* expression by antisense constructs or oligomers has been shown to result in differentiation and growth suppression in the absence of inducing agents (45-48). Induction of both reversible differentiation and, to a greater extent, terminal differentiation in H0-1 cells resulted in decreased *c-myc* expression. Downregulation of *c-myc* expression was independent of growth suppression, as indicated by the enhanced expression of *c-myc* in IFN- $\beta$  + IFN- $\gamma$  treated cells, even though this combination of agents resulted in maximum growth suppression without the induction of any morphologic or biochemical markers of melanoma differentiation. Based on the temporal relationship and the magnitude of *c-myc* downregulation by the various inducing agents, continued suppression of *c-myc* expression may be required for the induction of terminal differentiation in H0-1 cells. Studies using antisense *c-myc* constructs should prove valuable in directly addressing the relationship between *c-myc* expression and terminal differentiation in H0-1 cells.

Two immediate early response genes, *c-fos* and *c-jun*, code for transcription factors involved in nuclear signal transduction (35, 46, 47). Expression of these genes can be induced by many external stimuli, including cytokines, growth factors, serum, phorbol esters, neurotransmitters, and viral infection (35, 46, 47). The proteins *c-fos* and *c-jun* can form a heterodimer as part of the AP-1 transcription-factor complex that binds efficiently to

-80-

AP-1 sites (TGA<sup>c</sup>/cTCA) in DNA (35, 46, 47). Previous studies have indicated that both the *c-jun* and *c-fos* genes are activated during monocytic differentiation induced by TPA, macrophage colony-stimulating factor (M-CSF), and okadaic acid (48-50). Elevation of AP-1 activity also has been demonstrated during the induction of differentiation of F9 embryonal carcinoma stem cells by RA (51). In contrast the transcriptional enhancing activity of *c-jun*, *jun-B* (which is induced by a number of external stimuli that also induce *c-jun*) functions as a negative regulator of several genes normally activated by *c-jun* (52, 53). In the process of monocytic differentiation induced by TPA in human cells, *jun-B* gene transcription, steady-state mRNA levels, and mRNA stability are enhanced (54). Similarly, *jun-B* expression is enhanced during the process of monocytic differentiation induced in murine cells by serum-free conditioned medium from mouse lungs (55). Induction of growth suppression and both reversible and irreversible differentiation in HO-1 cells are unaltered at later time points. Unlike TPA-induced monocytic differentiation (48, 54), induction of *jun-B* expression by IFN- $\beta$  + MEZ in HO-1 cells is regulated only at the transcriptional level (56). These data indicate that enhanced expressions of *c-jun* and *jun-B* in HO-1 cells is not directly related to the induction of terminal differentiation in HO-1 cells. A sustained elevation of *c-jun* and *jun-B* expression, however, may be components of the differentiation program in HO-1 cells.

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The process of cellular differentiation is frequently associated with profound changes in cellular morphology that are related to cell-cell and cell-extracellular matrix interactions as well as the expression of cell



-81-

growth and cytoskeletal genes (57). In addition, cell shape and cell-extracellular matrix interactions also play important roles in the process of tumorigenesis and metastasis (58, 59). Transformed cells often exhibit

5 reductions in fibronectin expression as well as decreases in expression of specific integrin genes, which encode receptors for extracellular matrix proteins (60, 61). Of particular recent interest is the  $\alpha_5\beta_1$  integrin complex that appears to be the major receptor for fibronectin

10 (62). Decreases in  $\alpha_5$   $\beta_1$  expression have been found in oncogenically transformed cells (60) and overexpression of the combination of  $\alpha_5$  and  $\beta_1$  integrin cDNA in Chinese hamster ovary (CHO) cells results in a direct suppression of the transformed phenotype (61). Agents that induced

15 reversible differentiation (MPA + MEZ and RA + MEZ), irreversible differentiation (IFN- $\beta$  + MEZ), and increased growth suppression without inducing markers of differentiation (IFN- $\beta$  + IFN- $\gamma$ ) in HO-1 cells enhanced fibronectin,  $\alpha_5$  integrin, and  $\beta_1$  integrin expression.

20 These findings suggest that the specific combinations of cytokines, such as IFN- $\beta$  + IFN- $\gamma$ , resulting in growth suppression and combinations of agents that induce either a reversible commitment to differentiation or terminal differentiation in HO-1 cells, can directly modify

25 extracellular matrix and extracellular matrix receptor gene expression. The changes induced in these genes by these agents reflect a more normal, as opposed to the original, transformed cellular phenotype. In this context, it is also worth commenting on changes induced

30 tenascin expression as a consequence of treatment with the various differentiation-inducing and/or growth-suppressing agents. Tenascin is an extracellular-matrix protein expressed (or prominently expressed) in specialized embryonic tissues, cells of neuroectodermal

-82-

origin, and tumors (63). In general, tenascin is expressed at higher levels in undifferentiated vs. differentiated tumors (63). HO-1 cells express tenascin, and its level of expression is increased by IFN- $\gamma$ , alone or in combination with IFN- $\beta$ , whereas its expression is reduced by treatment with IFN- $\beta$ , MEZ, or RA or by continuous growth in the combination of IFN- $\beta$  + MEZ or RA + MEZ. These results provide further evidence that the continuous treatment of HO-1 cells for 96 h with specific differentiation-inducing agents can result in acquisition of a more differentiated cellular phenotype by these human melanoma cells.

Studies analyzing the mechanism of growth arrest during the process of differentiation in hematopoietic cells have implicated IFN- $\beta$  as an autocrine growth inhibitor important in this process (39, 64, 65). Supporting evidence for the involvement of autocrine IFN- $\beta$  in the differentiation process of hematopoietic cells include (1) the ability of IFN- $\beta$  antibody, but not IFN- $\alpha$  antibody, to partially block the reduction in c-myc mRNA and growth inhibition associated with the differentiation process; (2) the induction of interferon regulatory factor 1 (IRF-1), which is a positive transcription factor for expression of the IFN- $\beta$  gene, during the myeloid differentiation process; (3) the ability of IRF-1 antisense oligomers to partially block growth inhibition associated with IL-6 and leukemia inhibitory factor induction of differentiation; and (4) the induction of type I interferon (IFN- $\alpha/\beta$ ) gene expression during terminal differentiation in hematopoietic cells (39, 64, 65). A potential IFN- $\beta$  autocrine loop in the induction of specific programs of reversible and irreversible differentiation in human melanoma cells is also suggested by the experiments described in this article. Reversible

- 83 -

differentiation, resulting from treatment with MPA + MEZ and RA + MEZ, and terminal cell differentiation, resulting from growth in IFN- $\beta$  + MEZ, results in the enhanced expression of type I interferon-responsive genes, including HLA Class I antigen, ISG-15, and ISG-54. These same gene expression changes occur in HO-1 cells treated with conditioned medium obtained from IFN- $\beta$  + MEZ treated HO-1 cells. In addition, conditioned medium induces growth suppression in HO-1 cells and IFN- $\beta$  antibodies partially block the induction by conditioned medium of ISG-15 in HO-1 cells (56). Attempts to quantitate IFN- $\beta$  in conditioned medium from inducer-treated HO-1 cells have not been successful (66). A possible reason for the lack of quantifiable IFN- $\beta$  in HO-1 inducer-treated conditioned medium could be the presence of IFN- $\beta$  below the sensitivity of detection of the assay system, that is, level of IFN- $\beta$  below 2 U/ml. In this respect, the differentiating HO-1 system may be similar to hematopoietic cells induced to terminally differentiate by treatment with various inducers that also produce small quantities of high specific-activity autocrine IFN- $\beta$  (39). Further studies are required to characterize the putative autocrine IFN- $\beta$  produced by differentiating human melanoma cells and to determine its potential role in both the reversible commitment to differentiation and terminal differentiation in human melanoma cells. The present studies support the hypothesis that autocrine IFN- $\beta$  may also contribute to the differentiation process in solid tumors.

Analysis of gene expression changes resulting from exposure to IFN- $\beta$  + MEZ and conditioned medium from HO-1 cells treated with these inducers suggests the presence of additional autocrine factors produced during the differentiating process in HO-1 cells. One of these

-84-

putative autocrine factors is the previously identified melanoma growth factor termed MGSA (67). MGSA has been identified in the serum-free growth medium obtained from low-density cultures of the human malignant cell line Hs294T (67). The gene for MGSA has been cloned (68) and the deduced amino acid sequence for human MGSA is identical to that of the human "gro" cDNA isolated by Anisowicz et al. (69), now referred to as gro/MGSA. gro/MGSA is secreted by ~70% of primary cell cultures derived from human melanoma biopsies and by a majority of benign nevus cells with chromosomal abnormalities, whereas benign nevus cells with a normal karyotype are negative for MGSA production (70, 71). The level of gro/MGSA mRNA is enhanced in human melanoma cells treated with MGSA, indicating a potential autocrine function for this molecule (68); gro- $\alpha$  and gro- $\beta$  have also been shown to be primary response genes that are induced as a result of IL-1-mediated growth arrest in human melanoma cells (72). In addition, the expression and secretion of MGSA is strongly induced in other cell types, including human endothelial cells treated with a number of agents such as IL-1, TNF, lipopolysaccharide, thrombin, or TPA (73). These observations suggest that gro/MGSA production is not restricted to human melanoma cells and, in addition to stimulating the growth of specific melanoma cells, gro/MGSA may also play a role in the inflammation process. Applicants presently demonstrate that gro/MGSA gene expression is induced in HO-1 cells during specific programs of reversible differentiation and during terminal cell differentiation. In contrast, growth arrest, without the induction of biochemical or cellular markers of differentiation, does not result in gro/MGSA induction. The ability of conditioned medium obtained from IFN- $\beta$  + MEZ-treated HO-1 cells to induce gro/MGSA in naive HO-1 cells suggests that gro/MGSA may be produced

- 85 -

during the induction of terminal cell differentiation in HO-1 cells. At present the function of gro/MGSA (which is structurally related to a number of additional genes, including platelet factor-4,  $\beta$ -thromboglobulin, 5 connective tissue-activating peptide-3, and the murine KC gene) in melanoma development is not clear. The present data suggest, however, that in addition to its growth stimulatory effect on human melanoma cells, gro/MGSA may also play a role in melanoma cell differentiation.

10 In summary, the HO-1 cells culture system has been used to analyze the molecular changes associated with the reversible commitment to differentiation and terminal cell differentiation in human melanoma cells. Evidence 15 is presented indicating that induction of both processes may involve overlapping gene expression changes. However, the magnitude of the changes and the persistence of the changes suggest a potential involvement of defined programs of gene expression alterations in the induction 20 and maintenance of the terminal cells differentiation phenotype of human melanoma cells. Although their precise roles in melanoma cell growth and differentiation are not presently known, data are also presented that indicate that induction of differentiation results in the 25 production of autocrine factors, including IFN- $\beta$  and gro/MGSA. Further studies are required to define the functional significance of specific gene expression changes and specific autocrine factors in the process of terminal cells differentiation in human melanoma cells. 30 This information will be important in understanding the process of melanoma development and evolution and may result in the identification of novel target genes and molecules that could prove useful in the therapy of this neoplastic disease. In addition, the HO-1 35 differentiation model system appears ideally suited for

-86-

the identification and cloning of genes involved in the induction and maintenance of loss of proliferative capacity and terminal cell differentiation in human melanoma cells.

-87-

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Second Series of Experiments

Molecular biological approaches for the identification and cloning of genes displaying differential expression in both related and different cell types have been described (1-5). A particularly powerful procedure that has resulted in the identification of genes differentially expressed in diverse target cells is subtraction hybridization (4,5). This approach has been successfully used to identify genes that are specifically expressed during progression of the transformed/malignant phenotype (5,6), in cells undergoing growth arrest (7), induced by specific DNA damaging agents (8), expressed during specific stages of B cell development (9), and associated with programmed cell death (10). Subtraction hybridization is ideally suited for the identification of rare transcripts (4,5,9,11,14), or transcripts that exhibit small variations in expression between two cell types (4,8,11). As described in this article, subtraction hybridization is also an ideal procedure for identifying and cloning genes that are expressed at higher levels in cells induced to differentiate versus uninduced parental cells. In addition, by performing subtraction in the opposite direction, i.e., differentiation-inducer treated ( $Ind^+$ ) from untreated control ( $Ind^-$ ), the present protocol can also be used to identify genes that are suppressed during terminal differentiation.

Treatment of human melanoma cells with the combination of recombinant human fibroblast interferon (IFN- $\beta$ ) and the antileukemic compound mezerein (MEZ), results in a rapid cessation of cell growth and the induction of terminal cell differentiation, i.e., cells remain viable, but

-100-

they lose proliferative capacity (15-17). Terminal cell differentiation can be induced by IFN- $\beta$  plus MEZ in human melanoma cells either innately sensitive or resistant to either agent used alone (15,16). In contrast, treatment

5 of melanoma cells with either IFN- $\beta$  or MEZ alone results in a reversible alteration in differentiation phenotypes in the human melanoma cell line H0-1. This system represents a valuable experimental model for determining which changes in gene expression are correlated directly

10 with growth suppression as opposed to reversible differentiation and terminal cell differentiation. Applicants have presently developed a simple and effective subtraction hybridization protocol and used it to identify melanoma differentiation associated (*mda*)

15 genes displaying enhanced expression in cells treated with reversible- and terminal differentiation inducing compounds. Four types of *mda* genes have been identified, including genes upregulated by both IFN- $\beta$  and IFN- $\beta$  plus MEZ, both MEZ and IFN- $\beta$  plus MEZ, all three treatments

20 and only the combination of IFN- $\beta$  plus MEZ. This approach should prove amenable to other model systems resulting in the isolation of differentially expressed genes involved in important cellular processes.

-101-

## MATERIALS AND METHODS

## Cell Line and Differentiation Induction

5 The human melanoma cell line H0-1 is a melanotic melanoma derived from a 49-year-old female and was used between passage 125 and 150 (16). H0-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM-5) at 37°C in a 5% CO<sub>2</sub>-95% air humidified incubator. Cells were either untreated (Ind<sup>-</sup>) or treated (Ind<sup>+</sup>) with a combination of IFN- $\beta$  (2000 units per ml) and MEZ (10 ng per ml) for 2, 4, 8, 12 and 24 hr. For expression studies, H0-1 cells were untreated or treated for 12 and 24 hr with IFN- $\beta$  (2000 units per ml), MEZ (10 ng per ml) or IFN- $\beta$  plus MEZ (2000 units per ml plus 10 ng per ml) prior to isolation of cellular RNA and Northern blotting analysis (17).

## Construction of cDNA Libraries

20 Total cellular RNA from untreated (Ind<sup>-</sup>) and IFN- $\beta$  plus MEZ treated (2,4,8,12 and 24 hr) (Ind<sup>+</sup>) samples was isolated by the guanidinium isothiocyanate/CsCl centrifugation procedure and poly(A<sup>+</sup>) RNA was selected following oligo(dT) cellulose chromatography (18). cDNA synthesis was performed using the ZAP-cDNA™ synthesis kit from Stratagene® (La Jolla, CA) which is based on an adaptation of the Gubler and Hoffman method (19). A primer-adaptor consisting of oligo(dT) next to a unique restriction site (XhoI) was used for first strand synthesis. The double-stranded cDNAs were ligated to EcoRI adapters and then digested with the XhoI restriction endonuclease. The resultant EcoRI and XhoI cohesive ends allowed the finished cDNAs to be inserted into the  $\lambda$  ZAP II vector in a sense orientation with respect to the lac-Z promoter (20). The  $\lambda$  ZAP II vector

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-102-

contains pBluescript plasmid sequences flanked by bacteriophage-derived f1 sequences that facilitate in vivo conversion of the phage clones into the phagemid (20). Two cDNA libraries were constructed: a differentiation inducer-treated cDNA library (*Ind*<sup>+</sup>) (tester library); and a control uninduced cDNA library (*Ind*) (driver library). The libraries were packaged with Gigapack II Gold Packaging Extract (Stratagene®) and amplified on PLK-F' bacterial cells (Stratagene®).

#### Preparation of Double-Stranded DNA from *Ind*<sup>+</sup> Library

The *Ind*<sup>+</sup> cDNA phagemid library was excised from λ ZAP using the mass excision procedure described by Stratagene® (La Jolla, CA) [21]. Briefly, 1 X 10<sup>7</sup> pfu of *Ind*<sup>+</sup> cDNA library were mixed with 2 X 10<sup>8</sup> XL-1 Blue strain of Escherichia coli and 2 X 10<sup>8</sup> pfu of ExAssist helper phage in 10 mM MgSO<sub>4</sub>, followed by absorption at 37°C for 15 min (22). After the addition of 10 ml of LB medium, the phage/bacteria mixture was incubated with shaking at 37°C for 2 hr followed by incubation at 70°C for 20 min to heat inactivate the bacteria and the λ ZAP phage particles. After centrifugation at 4000 g for 15 min, the supernatant was transferred to a sterile polystyrene tube, and stored at 4°C before use.

To produce double-stranded DNA, 5 X 10<sup>7</sup> pfu of the phagemids were combined with 1 X 10<sup>9</sup> SOLR strain of Escherichia coli, which are nonpermissive for the growth of the helper phages and therefore prevent coinfection by the helper phages (22), in 10 mM MgSO<sub>4</sub>, followed by absorption at 37°C for 15 min. The phagemids/bacteria were transferred to 250 ml LB medium containing 50 µg/ml ampicillin and incubated with shaking at 37°C overnight. The bacteria were harvested by centrifugation, and the

-103-

double-stranded phagemid DNA was isolated by the alkali lysis method (18) and purified through a QIAGEN-tip 500 column (QIAGEN Inc., Chatsworth, CA).

5     **Preparation of Single-Stranded DNA from Control Ind Library**

The control Ind<sup>+</sup> cDNA library was excised from lambda ZAP using the mass excision procedure described above. The phagemid ( $5 \times 10^7$ ) were combined with  $1 \times 10^9$  XL-1 Blue strain of Escherichia coli in 10 mM MgSO<sub>4</sub>, followed by absorption at 37°C for 15 min. The phagemids/bacteria were transferred to 250 ml LB medium, and incubated with shaking at 37°C for 2 hr. Helper phage VCS M13 (Stratagene®, La Jolla, CA) was added to  $2 \times 10^7$  pfu/ml, and after incubation for 1 hr, kanamycin sulfate (Sigma) was added to 70 µg/ml. The bacteria were grown overnight. The phagemids were harvested and single-stranded DNAs were prepared using standard protocols (18).

20

**Pretreatment of Double- and Single-Stranded DNA Prior to Hybridization**

To excise the inserts from the vector, double-stranded DNA from the Ind<sup>+</sup> cDNA library was digested with EcoRI and XhoI, and extracted with phenol and chloroform followed by ethanol precipitation (5). After centrifugation, the pellet was resuspended in distilled H<sub>2</sub>O. Single-stranded DNA from Ind<sup>+</sup> cDNA library was biotinylated using photoactivatable biotin (Photobiotin, Sigma, St. Louis, MO) (23). In a 650 µl microcentrifuge tube, 50 µl of 1 µg/µl single-stranded DNA was mixed with 50 µl of 1 µg/µl photoactivatable biotin in H<sub>2</sub>O. The solution was irradiated with the tube slanted on crushed ice at a distance of 10 cm from a 300 watt sun lamp for

-104-

15 min. The DNA was further biotinylated by adding 25  $\mu$ l of photoactivatable biotin to the solution which was then exposed to an additional 15 min of irradiation as described above. To remove unlinked biotin, the reaction  
5 was diluted to 200  $\mu$ l with 100 mM Tris-HCl, 1 mM EDTA, pH 9.0, and extracted 3X with 2-butanol. Sodium acetate, pH 6.5 was added to a concentration of 0.3 M, and the biotinylated DNA was precipitated with two volumes of ethanol.

10

#### Subtracted Hybridization and Construction of Subtracted cDNA Library

Subtraction hybridization was performed essentially as described by Herfort and Garber (24) with minor  
15 modifications. In a 650  $\mu$ l siliconized microcentrifuge tube, 400 ng of EcoRI- and XhoI-digested *Ind*<sup>+</sup> cDNA library and 12  $\mu$ g of biotinylated *Ind*<sup>-</sup> cDNA library were mixed in 20  $\mu$ l of 0.5 M NaCl, 0.05 M HEPES, pH 7.6, 0.2% (wt/vol) sodium dodecyl sulfate and 40% deionized  
20 formamide. The mixture was boiled for 5 min and incubated at 42°C for 48 hr. The hybridization mixture was diluted to 400  $\mu$ l with 0.5 M NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA and then 15  $\mu$ g of streptavidin (BRL®) in H<sub>2</sub>O was added, followed by incubation at room temperature  
25 for 5 min. The sample was extracted 2X with phenol/chloroform (1:1), followed by back-extraction of the organic phase with 50  $\mu$ l of 0.5 M NaCl in TE buffer, pH 8.0. An additional 10  $\mu$ g of streptavidin was added and phenol/chloroform extraction was repeated. After  
30 removal of excess chloroform by brief lyophilization, the final solution was diluted to 2 ml with TE buffer, pH 8.0, and passed through a Centricon 100 filter (Amicon; Danvers, MA) 2X as recommended by the manufacturer. The concentrated DNA solution (approximately 50  $\mu$ l) was then



-105-

lyophilized. The subtracted cDNAs were ligated to EcoRI- and XhoI-digested and CIAP treated arms of the  $\lambda$ ZAP II vector and packaged with Gigapack II Gold packaging extract (Stratagene®, La Jolla, Ca). The library was  
5 then amplified using the PLK-F' bacterial cell.

#### Screening Subtracted cDNA Library

The mass excision of the library was performed using ExAssist helper phage as described above. The SOLR  
10 strain of Escherichia coli and cDNA phagemids were mixed at 37°C for 15 min and plated onto LB plates containing ampicillin and IPTG/X-gal. White colonies were chosen at random, isolated and grown in LB medium. Plasmid minireps and restriction enzyme digestions were performed  
15 to confirm the presence of inserts. The inserts were isolated and used as robes for Northern blotting analysis (5,25). Total cellular RNA was prepared from HO-1 cells treated with IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml), and IFN- $\beta$  plus MEZ (2000 units/ml plus 10 ng/ml),  
20 electrophoresed in 0.8% agarose gels and transferred to nylon membranes (Amersham, Arlington Heights, IL). Radiolabeled probes were generated by random oligonucleotide priming (25). Prehybridization, hybridization, posthybridization washes, and  
25 autoradiography were performed as described (5,18,25).

#### Sequencing of *mda* genes

The *mda* clones were sequenced using double-stranded pBluescript DNA as the template. DNA sequencing was  
30 performed using the Sanger dideoxynucleotide method with sequenase (United States Biochemical Corp., Cleveland, Ohio) and T3 promoter primer (GIBCO® BRL®, Gaithersburg, Md). This approach generates sequences from the 5' end of the inserts. Sequences were tested for homology to  
35 previously identified sequences using the GenBank FMBL

-106-

database and the GCG/FASTA computer program.

#### EXPERIMENTAL RESULTS

Subtraction hybridization represents a valuable methodology for isolating cDNA clones representing preferentially expressed mRNAs without prior knowledge of the selected gene or its encoded product (1-10). This procedure results in a substantial enrichment of differentially expressed cDNA clones and is often preferable to differential hybridization procedures using total cDNAs (4,5,11-14). A number of protocols have been reported for the generation of subtraction libraries [reviewed in 4]. The traditional approach involves hybridization of a first strand cDNA (tester) made from the mRNA of one cell type with mRNA (driver) prepared from a second cell type [or the first cell type treated with a specific gene modulating agent(s)] [7-9]. Single-stranded unhybridized cDNAs are then selected by hydroxylapatite column chromatography and they are used as templates for the synthesis of second-strand cDNA (7-9). However, this procedure has a number of limitations, including the requirement for RNA handling during hybridization, which can be problematic, and the limited quantity of cDNAs recovered following hybridization and column chromatography. Other subtraction hybridization protocols involve hybridization of cDNA and photobiotinylated RNA (23,26). Problems may still arise because of the requirement for large amounts of mRNA and from manipulation of RNA during the hybridization procedure (22). Recent improvements in subtraction hybridization utilize cDNA libraries as both tester and driver nucleic acid populations (24,27,28). By using driver sequences present in cloned forms, the newer approaches circumvent the problems associated with insufficient quantities of mRNAs or difficulties

-107-

resulting during the preparation and manipulation of mRNAs. Improvements in subtraction hybridization procedures have included: the use of phagemid subtraction hybridization (27); the use of single-stranded phagemids with directional inserts (28); and the use of double-stranded cDNA inserts as tester and single-stranded cDNAs as the driver (21).

The procedure applicants have used to construct subtraction libraries involves a modification of the protocols described by Rubenstein et al. (28) and Herfort and Garber (24). This strategy is outlined in Figure 8. Applicants' approach to subtraction library construction uses  $\lambda$  phage and commercially available reagents. In other similar procedures (22,27,28), the end products of subtraction hybridization are either single-stranded phagemid DNA, which is converted to double-stranded DNA, or double-stranded inserts, which are ligated to plasmid vectors. These procedures have two potentially limiting drawbacks including, the lower efficiency of bacterial transformation with plasmids versus phage infection and the need for special precautions to remove the double-stranded phagemids contaminating the driver single-stranded DNA preparation. By using  $\lambda$  phage as vectors, these problems are easily avoided. The efficiency of phage infection of bacteria is high, often attaining levels of  $10^9$  PFU/ $\mu$ g DNA (21). In addition, problems with contaminating plasmids in the preparation are also eliminated because they will not be packaged and transfected into bacteria. This approach, therefore, results in the construction of subtraction libraries of high titer. By employing  $\lambda$  Uni-ZAP vectors which can be converted into phagemids by in vivo excision, the laborious work of subcloning the DNA inserts into

-108-

plasmids is unnecessary.

5 cDNA libraries and subtraction libraries are prepared using the commercial ZAP-cDNA™ synthesis kit from Stratagene® (La Jolla, CA) (5). This product has several advantages for the construction of subtraction libraries. First, the XhoI adapter-primer permits the cDNA to be inserted into the vector in a unidirectional orientation. The efficiency of subtraction hybridization will be high  
10 if hybridization occurs only between complementary molecules in the different cDNA libraries instead of complementary molecules in the same cDNA library. This improved subtraction hybridization is achieved by using both single-stranded and double-stranded unidirectional  
15 cDNA libraries from each experimental condition. For construction of *mda* subtraction libraries (Figure 8), both the H0-1 control *Ind*<sup>+</sup> and the IFN- $\beta$  plus MEZ treated *Ind*<sup>+</sup> cDNA libraries were constructed in a unidirectional manner. The efficiency of subtraction hybridization was  
20 insured by using single-stranded unidirectional *Ind*<sup>+</sup> cDNA as the driver. Secondly, the bacteriophage f1 origin of replication, which is present in the  $\lambda$  ZAP II vector, permits excision of pBluescript II SK(-) phagemids from the bacteriophage and rescue of single-stranded DNA with  
25 the assistance of helper phage (20). The Escherichia coli strains XL-1 blue and SOLR, which are provided as part of the ZAP-cDNA™ kit, are very useful in preparing single-stranded and double-stranded phagemid DNA. The XL-1 blue strain is permissive for ExAssist helper phage growth, while the SOLR strain is nonpermissive (22). The  
30 phagemids are excised with ExAssist helper phage in XL-1 blue bacterial cells. The phagemids are then grown in SOLR bacterial cells for harvesting double-stranded DNA or in XL-1 blue cells with helper phages for harvesting

-109-

single-stranded DNA. Using this approach, contamination of helper phage and single-stranded DNA in the double-stranded DNA preparation was minimized. Contamination could decrease the efficiency of subtraction hybridization because of the complementary binding between the single-stranded cDNA and any potentially unique sequences from the same cDNA library (tester). This could potentially result in a failure of the unique sequences to form double-stranded inserts with appropriate ends which can be ligated into the vectors. A third consideration is the commercial availability of Uni-ZAP arms which can be used as vector for the construction of subtraction libraries. Tester inserts are released from the phagemid vector by digestion with the restriction enzyme EcoRI and XhoI. After subtraction hybridization, the remaining inserts which are in the double-stranded form because of complementary hybridization are ligated in a unidirectional manner into Uni-ZAP arms because of the EcoRI and XhoI cohesive ends. This approach eliminates the requirement for additional vectors. The subtraction library is then converted into a phagemid library which can be easily manipulated for screening, sequencing, in vitro RNA transcription, and mutagenesis. Without the advent of well designed commercial kits, subtraction hybridization and subtraction library construction is both time and labor intensive. The procedure applicants describe in this article is straight forward and highly efficient in producing subtraction libraries.

30

Employing the approaches applicants describe above, cDNA libraries from control H0-1 cells (Ind<sup>-</sup>) and H0-1 cells treated with the terminal differentiation inducing agents IFN- $\beta$  plus MEZ (Ind<sup>+</sup>) have been constructed. The

-110-

original titers of the cDNA libraries were  $1.2 \times 10^6$  PFU and  $1.7 \times 10^6$  PFU for the *Ind* and *Ind*<sup>+</sup>, respectively. The high titers obtained suggest that the cDNA libraries are representative of the mRNAs produced under the experimental conditions used. The purity of the single-stranded and double-stranded DNA was examined by digestion with the restriction endonucleases EcoRI and XhoI. Unlike double-stranded DNA, the single-stranded DNA could not be digested with the restriction endonucleases. This is demonstrated in Fig. 9, in which plasmid vector is released after digestion of double-stranded DNA but not single-stranded DNA. Four hundred ng of double-stranded DNA (tester:*IND*<sup>+</sup>) and 12 µg of single-stranded DNA (driver:*Ind*) were used for subtraction hybridization. After a single-round of hybridization, the *Ind*<sup>+</sup> subtraction library was constructed in Uni-ZAP XR vector with an original titer of 8 to  $10 \times 10^3$  PFU. Additional rounds of subtraction hybridization resulted in a low percentage of colonies which contained inserts. This may result because of the low concentration of potentially unique sequences remaining after the first round of subtraction hybridization. This observation indicates that the subtraction hybridization protocol applicants have utilized is very efficient and the requirement for additional subtraction hybridizations may not be necessary to identify differentially expressed genes.

Screening of subtraction libraries for differentially expressed sequences can be achieved using several procedures. In a number of studies, subtraction libraries are screened using differential hybridization techniques (7, 8, 27). However, the sensitivity of this procedure is limited by the relative abundance of the

-111-

target mRNA. The enrichment of target sequences obtained in our subtraction libraries permitted the random isolation of clones for evaluation of mRNA expression in undifferentiated H0-1 cells or H0-1 cells treated with IFN- $\beta$ , MEZ or IFN- $\beta$  + MEZ. After in vivo excision, bacteria containing the subtraction library were plated and randomly isolated clones were used to prepare plasmids. The EcoRI/XhoI digested cDNA inserts from these clones were then used as probes for Northern blotting analysis of mRNA expression under the different experimental conditions. Among 70 cDNA clones initially analyzed, 23 clones were found to display differences in gene expression between *Ind* and *Ind*<sup>+</sup> treated H0-1 cells. As expected, subtraction of control H0-1 cDNAs from IFN- $\beta$  plus MEZ treated H0-1 cDNAs results in a series of *mda* genes which displayed enhanced expression after 24 hr treatment with the inducer. These included *mda* genes which were inducible by both IFN- $\beta$  and IFN- $\beta$  plus MEZ, i.e. *mda*-1 and *mda*-2; by both MEZ and IFN- $\beta$  plus MEZ, i.e., *mda*-3; by IFN- $\beta$ , MEZ and IFN- $\beta$  plus MEZ, i.e., *mda*-4; and uniquely by IFN- $\beta$  and MEZ, i.e., *mda*-5 and *mda*-6 (Fig. 10). Specific *mda* genes also displayed elevated expression after 96 hr exposure to IFN- $\beta$  plus MEZ (data not shown).

Of the six *mda* genes reported in this study, only *mda*-3 corresponds to a previously reported gene (Fig. 11). At present, 245 bp of *mda*-3 have been sequenced and this cDNA shares >99% homology with the reported sequences of pLD78 (29), pAT 464 (30,31), pAT 744 (31) and GOS19 (32-34). The pLD78 cDNA is inducible by either TPA or a T-cell mitogen, phytohemagglutinin (PHA), in human tonsillar lymphocytes (29). *mda*-3 is induced in H0-1 cells within 24 hr of treatment with MEZ, IFN- $\beta$  plus MEZ and IFN- $\beta$

-112-

plus TPA (data not shown). The sequence of the 5' flanking region of the genomic DNA encoding for the pLD78 cDNA displayed a significant homology with corresponding regions of the human interleukin 2 and immune interferon genes (29). pAT 464 and pAT 744 are inducible by TPA and PHA, with maximal induction resulting from the combination of agents, in T-cells, B-cells and the promyelocytic cell line HL-60 (31). In contrast, these cDNAs are not expressed in human fibroblasts, although as indicated in the present study a potentially similar cDNA, *mda-3*, is inducible in human melanoma. pAT 464 and pAT 744 share some critical amino acid similarity with a family of secreted factors including connective tissue activating factor III, platelet factor 4, an IFN- $\gamma$ -induced factor, macrophage inflammatory protein and a factor chemotactic to neutrophils (3-10C, monocyte-derived neutrophil chemotactic factor, neutrophil-activating factor) (31). GOS19 genes are members of the "small inducible" family of genes, which exhibit similar exon-intron organizations and which encode secreted proteins with similar organization of cysteine and proline residues (32-34). The GOS19-1 mRNA is enhanced rapidly by the addition of both cycloheximide or lectin to cultured human blood mononuclear cells (32). This cDNA has sequence homology to the murine gene that encodes an inhibitory cytokine (MIP1 $\alpha$ /SCI) which decreases stem cell proliferation (32). In this context, GOS19-1, which is the main GOS19 gene expressed in adult T lymphocytes, may encode a homeostatic negative regulator of marrow stem cell populations. The role of *mda-3* in the process of melanoma cell growth and differentiation remains to be determined.

Studies are currently in progress to further characterize the novel *mda* genes, *mda-1*, *mda-2*, *mda-4*, *mda-5*, and *mda-*



-113-

6, and determined their expression in different stages of melanoma evolution and during the induction of growth suppression, the reversible commitment to differentiation and the induction of terminal differentiation in human melanoma cells. It should be emphasized that the cDNA clones applicants have currently analyzed represent only a small percentage of the complete subtraction library. This suggests that this subtraction library has the potential for identifying and cloning additional genes involved or associated with the chemical induction of differentiation and growth suppression in human melanoma cells. In addition, by altering the driver DNA, i.e., using combinations of cDNA libraries constructed from H0-1 cells treated singularly with IFN- $\beta$  and MEZ, it should be possible to further enrich for gene(s) uniquely expressed in terminally differentiated human melanoma cells, i.e. those treated with the combinations of IFN- $\beta$  + MEZ.

20 In summary, applicants presently describe an efficient and sensitive procedure for the production of subtraction hybridized cDNA libraries which can be used for the identification and cloning of differentially expressed genes. The basic protocol utilizes biotinylated single-stranded DNA as the driver and bacteriophage as the vector and relies on the availability of commercial reagents for construction of subtraction cDNA libraries. The usefulness of the current protocol is demonstrated by the high level of enrichment obtained from genes in the subtracted library associated with the induction of differentiation of human melanoma cells, i.e., *mda* genes. This procedure should find wide applicability for the identification and cloning of differentially expressed genes. These can include, but are not limited to, genes displaying modified expression between closely related

-114-

cell types, between disparate cell types, in cells induced to lose proliferative ability or undergo apoptosis, in cells treated with chemotherapeutic agents, and in cells induced or committed to reversible or

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-115-

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-121-

Third Series of Experiments

5 Development of malignant melanoma in humans, with the  
exception of nodular type melanoma, is a progressive  
process involving a discrete series of well defined  
stages. Although the focus of intensive scientific  
scrutiny, the genetic elements controlling melanocytic  
conversion into the various stages of the evolving  
10 melanoma have not been identified. In addition, no  
consistently effective therapy is currently available to  
treat metastatic melanoma. The long-term goal of the  
present proposal is to define the genes regulating  
melanoma growth, differentiation and progression. This  
15 information could prove valuable in elucidating potential  
targets for therapeutic intervention.

Tumor progression in melanocytes is associated with  
altered patterns of normal melanocytic differentiation.  
Chemical induction of terminal differentiation in tumor  
20 cells represents a useful approach for reversing the  
negative prognosis associated with specific neoplasms.  
Recent studies indicate that the specific combination of  
recombinant human fibroblast interferon (IFN- $\beta$ ) and the  
antileukemic compound mezerein (MEZ) can reprogram human  
25 melanoma cells to undergo terminal differentiation, i.e.,  
cells retain viability but they irreversibly lose  
proliferative capacity. In contrast, application of  
comparable doses of IFN- $\beta$  or MEZ alone to human melanoma  
cells results in a reversible commitment to  
30 differentiation, i.e., removal of the inducing agent  
results in the resumption of cell growth and the loss of  
specific differentiation-associated properties.

Subtraction hybridization is used to identify the  
35 genotypic changes associated with induction of terminal

-122-

differentiation in human melanoma cells. Using this approach, cDNAs displaying enhanced expression in melanoma cells induced to terminally differentiate versus untreated melanoma cells have been identified. Partial  
5 sequence analysis of these differentially expressed cDNAs, tentatively called melanoma differentiation associated (*mda*) genes, indicate that they consist of both known and previously unidentified genes. Specific  
10 *mda* genes may represent novel genetic elements involved in tumor cell growth and/or commitment of cells to the melanocyte lineage.

The specific aims of this proposal are to characterize and determine the functional roles of the *mda* genes in melanoma growth, differentiation, and progression. With  
15 these aims in mind studies will be conducted to:

- 1) Determine the pattern and regulation of expression of the *mda* genes in melanocytes, nevi, radial growth phase melanoma, vertical  
20 growth phase melanoma and metastatic melanoma cells;
- 2) Analyze the relationship between *mda* gene expression and the induction of reversible  
25 commitment to differentiation, growth suppression without the induction of differentiation, DNA damage and stress responses and induction of terminal differentiation in human melanoma and other  
30 model differentiation systems;
- 3) Isolate full-length cDNAs of *mda* genes that may be involved in melanoma differentiation or progression and directly determine their  
35 potential functional role in differentiation

-123-

and progression of human melanoma;

- 4) Isolate and characterize the promoter region of appropriate *mda* genes and analyze their regulation in human melanocytes, nevi and melanoma.

Experimental strategies designed to activate genes mediating a loss of proliferative capacity and the reprogramming of melanoma cells to terminally differentiate, may represent novel approaches for effective therapeutic intervention in metastatic melanoma. Elucidation of the function of the cloned *mda* genes should provide molecular insights into the process of melanoma differentiation and progression. In addition, specific *mda* genes may represent targets of clinical interest which can be exploited for suppressing the growth of metastatic melanoma and other tumorigenic cell types.

20

#### Background and Significance

Malignant melanoma epitomizes the process of tumor progression and emphasizes the selective nature of the metastatic phenotype and the growth dominant properties of metastatic cells (rev. 1 to 3). Of the numerous types of cancer developing in North American populations, melanoma is increasing at the fastest rate and it is estimated that as many as 1 in 100 currently born children may eventually develop superficial spreading type melanoma (3,4). Although melanoma is readily curable at early stages, surgical and chemotherapeutic interventions are virtually ineffective in preventing metastatic disease and death in patients with advanced stages of malignant melanoma. These observations

-124-

emphasize the need for improved therapeutic approaches to more efficaciously treat patients with metastatic melanoma.

5 Development of malignant melanoma in humans, with the exception of nodular type melanoma, consists of a series of sequential alterations in the evolving tumor cells (rev. 1-4). These include conversion of a normal melanocyte into a common acquired melanocytic nevus  
10 (mole), followed by the development of a dysplastic nevus, a radial growth phase (RGP) primary melanoma, a vertical growth phase (VGP) primary melanoma and ultimately a metastatic melanoma. As indicated above, although readily treatable during the early stages of  
15 development even during the VGP if the lesion is  $\leq$  0.76-mm thick, currently employed techniques are not very effective (<20% survival) in preventing metastatic spread and morbidity in patients with VGP lesions  $>$  4.0-mm thickness. This experimental model system is ideally  
20 suited to evaluate the critical genetic changes that mediate both the early and late phases of melanoma evolution.

A less toxic approach to cancer therapy involves a  
25 process termed differentiation therapy (5-9). Two premises underlie this therapeutic modality. (A) Many neoplastic cells display aberrant patterns of differentiation resulting in unrestrained growth; and (B) Treatment with the appropriate agent(s) can result in the  
30 reprogramming of tumor cells to lose proliferative capacity and become terminally differentiated. Intrinsic in this hypothesis is the assumption that the genes that mediate normal differentiation in many tumor cells are not genetically defective, but rather they fail to be  
35 appropriately expressed. The successful application of

-125-

differentiation therapy in specific instances may result because the appropriate genes inducing the differentiated phenotype become transcriptionally activated resulting in the production of appropriate gene products required to induce terminal cell differentiation. Applicants have tested this hypothesis using human melanoma cells (10-14). Treatment of human melanoma cells with the combination of recombinant human fibroblast interferon (IFN- $\beta$ ) and the antileukemic compound mezerein (MEZ) results in a rapid cessation of growth, an induction of morphological changes, an alteration in antigenic phenotype, an increase in melanin synthesis and an irreversible loss in proliferative capacity, i.e., terminal cell differentiation (10,11,14). IFN- $\beta$  plus MEZ effectively induce terminal differentiation in human melanoma cells innately resistant to the antiproliferative effect of either agent used alone (10). In contrast, IFN- $\beta$  or MEZ applied alone induce a number of similar biochemical and cellular changes in human melanoma cells, however, these changes are often reversible following removal of the inducing agent, i.e., reversible commitment to differentiation (10,14). Although the effect of IFN- $\beta$  plus MEZ toward normal human melanocytes has not been reported, Krasagakis et al. (15) did determine the effect of IFN- $\beta$  plus TPA (which was present in the melanocyte growth medium) on the growth of normal human melanocytes. MEZ shares a number of in vitro properties with TPA, including its ability to replace TPA for the growth of normal melanocytes, to activate protein kinase C and to modulate cell differentiation (14,16). In contrast to TPA, however, MEZ is a very weak tumor promoter when substituted for TPA in the initiation-promotion model of carcinogenesis on mouse skin, although it is quite potent during the second phase of tumor promotion (17,18). When normal

-126-

melanocytes are grown under optimal growth conditions, including TPA, cholera toxin, isobutylmethylxanthine and fetal bovine serum, even high doses (10,000 units/ml) of leukocyte (IFN- $\alpha$ ), fibroblast (IFN- $\beta$ ) or immune (IFN- $\gamma$ ) interferon does not inhibit growth (15). In contrast, when grown in modified melanocyte medium not containing TPA and resulting in reduced growth potential, only IFN- $\beta$  significantly inhibits proliferation. When tested in serum-free medium, all three interferon preparations are growth inhibitory toward the SKMel-28 human melanoma cell line, with IFN- $\beta$  again being the most growth-suppressive (15). IFN- $\beta$  has been shown to be more growth suppressive than IFN- $\alpha$  toward several additional human melanomas grown in serum containing medium (10). These results support the hypothesis that IFN- $\beta$  may be a negative-regulator of melanocyte proliferation and malignant transformation results in an increased sensitivity to interferons (10,15). In the case of TPA, it is an obligatory requirement for the in vitro growth of normal melanocytes, whereas TPA and MEZ are growth inhibitory toward many human melanoma cells (14-16,19,20).

Melanoma represents a useful experimental model to analyze the process of tumor progression (rev 1-3). Cell culture systems are available that permit the growth of normal melanocytes, nevi and melanoma cells representing different stages of tumor progression (1-3,20-25). Analyses of the properties of cells of the melanocyte lineage indicate a number of traits that allow the different stages in melanoma evolution to be distinguished. These include: (a) morphology;; (b) life span in culture; (c) chromosomal abnormalities; (d) anchorage-independent growth; (e) tumorigenicity; (f) expression of HLA-DR (class II HLA antigens) and

-127-

intercellular adhesion molecule-1 (ICAM-1) antigens; (g) response to the tumor promoting agent 12-0-tetradecanoyl-phorbol-13-acetate (TPA); (h) growth factor independence in vitro; (i) autocrine production of basic fibroblast growth factor (bFGF) and (j) growth inhibition by cytokines (rev 2,21,25). A limitation of the melanoma progression model, however, is the inability to obtain from the same patient who has developed a primary RGP or an early VGP melanoma (less than 0.76 mm in thickness), a genetically related more progressed melanoma. Recent studies by Dr. Kerbel and colleagues (25) suggest that by appropriate manipulation (use of matrigel) and tumor selection in nude mice, it may be possible to spontaneously progress early-stage, non- or poorly tumorigenic (in nude mice) human melanoma cell lines to a more progressed tumorigenic and metastatic state. In addition, Dr. Albino and colleagues (20) demonstrated that normal human melanocytes could be progressed to a complete melanoma phenotype and genotype following infection with a retrovirus containing the viral Ha-ras oncogene. Transformed melanocytes acquired the full spectrum of melanoma properties and displayed the same cytogenetic changes occurring during melanoma development in vivo (20). These cell lines will prove useful for evaluating the biochemical and genetic changes involved in melanoma progression. In summary, the ability to clearly define specific components of melanoma evolution will provide a valuable experimental model to define the genotypic changes mediating tumor progression.

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The critical genomic changes that mediate melanoma development and progression remain to be defined. Recent studies have addressed the potential relationship between the expression of specific oncogenes, growth factor genes

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-128-

(in addition to basic fibroblast growth factor (bFGF)), growth factor receptor genes, protease genes and early response genes and melanoma progression (26-30). Using a panel of metastatic melanoma cell lines, steady state mRNA transcripts for several growth factors (bFGF, platelet-derived growth factor (PDGF)-A, PDGF-B, transforming growth factor (TGF)- $\beta_1$ , TGF- $\alpha$ , melanoma growth-stimulating activity (MGSA; also called gro), interleukin (IL-1 $\alpha$  and IL-1 $\beta$ ) and early response (c-fos, c-jun and jun-B) genes have been found (27, 28). All of the metastatic melanoma cell lines expressed the bFGF gene and the majority of metastatic melanoma expressed c-fos, c-jun and jun-B in both serum-free and serum containing medium. With respect to the other growth factor genes tested, each metastatic melanoma displayed a pattern of expression that was specific and different (27). In contrast, two strains of normal melanocytes expressed TGF- $\beta_1$  but not bFGF, PDGF, TGF- $\alpha$  or MGSA mRNA at detectable levels (27). Although metastatic melanoma and normal melanocytes express c-fos, c-jun and jun-B, the expression of these transcripts in normal melanocytes was dependent on the presence of growth-promoting agents in the medium (28). In contrast, different levels of the early response genes were observed in metastatic melanoma cells grown in the presence or absence serum (28). In general, an increase in jun-B and c-fos RNA transcripts and a decrease in c-jun RNA transcripts were observed in metastatic melanomas compared to neonatal melanocytes (28). The relevance of these differences in early response gene expression in metastatic melanomas compared to neonatal melanocytes remains to be determined. In a recent study, Albino et al. (30) used PCR to determine the level of RNA transcripts for 11 different growth factors in 19 metastatic human melanoma cell lines and 14



-129-

normal human foreskin melanocyte cell lines. Transcripts for TGF- $\beta_2$  (19 of 19), TGF- $\alpha$  (18 of 19) and bFGF (19 of 19) were found in metastatic melanoma but not in the normal melanocytes. In contrast, TGF- $\beta_1$  and TGF- $\beta_3$  were expressed in both metastatic melanoma and normal melanocytes. The significance of these changes to melanoma progression is not apparent. These results suggest, however, that the differential expression of specific genes, i.e., bFGF, TGF- $\beta_2$ , TGF- $\alpha$  and possibly early response genes, may contribute to or may be directly related to the metastatic melanoma phenotype.

On the basis of genetic linkage analysis of familial melanoma, cytogenetic analysis, and various molecular techniques (including RFLP analysis to identify LOH in tumor DNA samples and microcell gene transfer procedures) it is now apparent that nonrandom changes in genes on chromosomes 1, 6, 7 and 9 may contribute to the etiology of human melanoma (31-41). At this stage of analysis, at least 5 genes, mapping to chromosomes 1, 6, 7 and 9, appear to contribute to the development of malignant melanoma, and extensive tumor heterogeneity also implicates additional loci as contributors to the malignant phenotype (rev. 38,41). A proposed model of tumor progression from melanocyte to metastatic melanoma suggests that alterations in chromosome 1 and 9 are early events in melanoma progression, whereas changes in chromosome 6 and 7 represent later stages of tumor progression (38,41). A direct demonstration of the suppressive role of chromosome 6 in human melanoma has recently been demonstrated (37). Employing microcell mediated gene transfer, a normal human chromosome 6 was inserted into human melanoma cells (UACC-903) and shown to suppress transformed properties in vitro and

-130-

tumorigenic potential in nude mice (37). Dr. Welch and colleagues have also demonstrated that insertion of a normal human chromosome 6 into the C8161 human melanoma cell line results in a suppression of metastatic potential, but not tumorigenic potential (42). The apparent discrepancy between the results of Trent et al. (37) and Welch et al. (42) may relate to differences between UACC-903 and C8161 cells. C8161 cells exhibit both tumorigenic and metastatic properties in nude mice, whereas UACC-903 cells are tumorigenic but not metastatic in nude mice. This difference might mask the presence of a metastasis suppressor on chromosome 6 or alternatively might suggest that chromosome 6 contains both a tumor and a metastatic suppressor gene. The chromosome 6 containing C8161 cells have also been found to differ from parental C8161 cells in their biological response and in gene expression after treatment with IFN- $\beta$  plus MEZ (43). Further studies are required to determine if a similar suppression of transformed and tumorigenic properties can be induced by reintroduction by microcell mediated transfer of chromosomes 1, 7 and/or 9 into melanoma cells containing abnormalities in these chromosomes. Similarly, the mechanism by which the putative melanoma suppressor gene(s) on chromosome 6, as well as suppressor gene(s) located on additional chromosomes, exert their effects on human melanoma cells and how these genes regulate tumor progression remain to be determined.

Recent studies have demonstrated the existence of at least 23 IFN- $\alpha$  genes and pseudogenes, all of which reside proximal to the IFN- $\beta$  gene that is located on locus 9p (9p22-13) (44,45). Nonrandom alterations in specific loci on chromosome 9 appear to be an early event in melanoma evolution. It is intriguing, therefore, that

-131-

90% (9/10) of informative melanoma DNAs have shown a reduction for one to five of the loci tested in the same region as the IFN- $\alpha/\beta$  gene (41) including a 2 to 3 megabase region on 9p21 in which a putative melanoma tumor-suppressor gene appears to be located (40). Similarly, homo- or homozygously deleted  $\alpha$ - and  $\beta$ -interferon genes have been found in human acute lymphoblastic leukemias and human malignant gliomas (44-47). This observation is interesting, since interferons display antiproliferative activity toward both human melanoma and lymphoblastic leukemic cells and can be viewed therefore as tumor suppressor proteins (rev. 13). This data is compatible with the hypothesis that a tumor suppressor locus for both melanoma and leukemia is located on chromosome 9 and tumor suppression may in specific cancers involve alterations in the interferon gene region.

The mechanism by which the combination of IFN- $\beta$  + MEZ induces a rapid irreversible inhibition in cellular proliferation and terminal differentiation in human melanoma cells remains to be determined. Since actinomycin D and cycloheximide can inhibit the induction of morphologic changes, growth suppression and the induction of differentiation in H0-1 cells induced by IFN- $\beta$  + MEZ (49), transcriptional activation or suppression of specific gene(s) following treatment with these agents may be the primary determinants of induction of differentiation. A modified subtraction hybridization procedure was used to identify and characterize the critical genes that mediate and which are associated with the chemical induction (49). Using this approach, a series of cDNAs have been identified, termed melanoma differentiation associated (mda) genes, which display enhanced expression in terminally differentiated human

-132-

melanoma cells (49). Specific cDNAs have been identified which represent novel genes, i.e., their sequences have not been described previously in any of the DNA data bases. By using appropriate sense and anti-sense oligomers and expression constructs, studies will be conducted to determine the functional role of the *mda* genes in melanoma growth, differentiation and progression. In addition, by employing human melanoma cells representing specific stages in melanocytic evolution to metastatic melanoma it will also be possible to address the relationship between states of tumor progression and susceptibility to induction of terminal differentiation. An understanding of the process of terminal cell differentiation and the function of the *mda* genes could prove useful in defining the molecular basis of melanoma progression and in designing improved strategies for the therapy of malignant melanoma and other cancers.

20 A. Induction of Terminal Differentiation in Human Melanoma Cells by IFN- $\beta$  plus MEZ.

As discussed in Background and Significance, a hallmark of many cancers is an inability to undergo normal programs of cellular differentiation. If this assumption is correct, and if the genetic machinery of the tumor cells could be reprogrammed to regain their commitment to normal differentiation, then appropriate external stimuli could be employed to induce a loss of proliferative capacity and terminal differentiation (5-14). In studies designed to directly test this hypothesis, applicants have successfully induced terminal differentiation in human melanoma cells with the combination of recombinant IFN- $\beta$  and the antileukemic compound mezerein (MEZ) (10,11,14). In contrast, the combination of recombinant

-133-

leukocyte interferon (IFN- $\alpha$ ) and MEZ resulted in a potentiation of growth suppression, but terminal differentiation was not induced, i.e., treated cells retained proliferative capacity (10). The combination of IFN- $\beta$  plus MEZ was effective in inducing terminal differentiation in human melanoma cells relatively resistant or sensitive to the growth suppressive effects of either agent employed alone (10). Induction of terminal differentiation in the human melanoma cell line H0-1 by continuous exposure for 4 or 7 days to IFN- $\beta$  plus MEZ was associated with: (a) a rapid, within 24 hr, inhibition in proliferation (Fig. 12) (10,11); (b) a profound alteration in cellular morphology (treated cells displayed dendrite-like processes) (Fig. 13) (10); and (c) an induction (in melanotic melanoma) or an increased synthesis (in melanotic melanoma) of melanin, a marker of melanoma cell differentiation (10). By employing varying doses of IFN- $\beta$  and MEZ and different treatment schedules (24 hr, 4 days and/or 7 days), it has been possible to separate the chemical-induction of melanoma differentiation into three stages. These include an early completely reversible-induction phase (low doses of inducing agents for 4 or 7 days), a late partially reversible-induction phase (higher doses of inducing agents for 4 days), and an irreversible terminal-differentiation phase (specific doses of inducing agents for 24 hr, 4 days or 7 days)) (10-14).

In contrast to IFN- $\beta$  plus MEZ that induces an irreversible loss in proliferative capacity and terminal differentiation in the human melanoma cell line H0-1, the combination of IFN- $\beta$  plus IFN- $\gamma$  induces enhanced growth suppression without terminal differentiation (12, 14, 50). In addition, IFN- $\beta$  plus IFN- $\gamma$  also fail to induce an increase in melanin synthesis in H0-1 cells (50).

-134-

When treated with trans retinoic acid (RA), both melanin levels and tyrosinase levels are increased in H0-1 cells, but growth is not suppressed (14,51). Exposure to 3  $\mu$ M mycophenolic acid (MPA) for 96 hr results in growth inhibition, morphologic changes, enhanced melanin synthesis and enhanced tyrosinase activity in H0-1 cells (14, 51). However, these affects are reversible when H0-1 cells treated with 3  $\mu$ M MPA for 4 days are then grown in the absence of MPA for an additional 7 days (14). These results suggest that at the dose- and time-interval used, MPA (alone or in combination with MEZ) induces a reversible-induction of differentiation in H0-1 cells and not terminal differentiation. The effect of different agents on growth and the properties of H0-1 cells is summarized in Table 2 (14).

-135-

TABLE 2

	Experimental Conditions <sup>a</sup>	Morphology changes <sup>b</sup>	Melanin Synthesis <sup>c</sup>	Tyrosinase activity <sup>d</sup>
5	RA (2.5 $\mu$ M)	-	1+	2+
	MPA (3.0 $\mu$ M)	+	2+	3+
10	MEZ (10 ng/ml)	+	1+	NT
	IFN- $\beta$ (2000 U/ml)	-	1+	NT
15	IFN- $\gamma$ (2000 U/ml)	-	-	NT
	RA + MEZ (2.5 $\mu$ M + 10 ng/ml)	+	NT	NT
20	MPA + MEZ (3.0 $\mu$ M + 10 ng/ml)	+	NT	NT
25	IFN- $\beta$ + IFN- $\gamma$ (1000 U/ml + 1000 U/ml)	-	1+	NT
30	IFN- $\beta$ + MEZ (2000 U/ml + 10 ng/ml)	+	4+	NT
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-136-

TABLE 2 (Continued)

	Experimental conditions <sup>a</sup>	Growth Suppression (reversible) <sup>c</sup>	Terminal cell differentiation <sup>f</sup>
5	RA (2.5 $\mu$ M)	-	-
	MPA (3.0 $\mu$ M)	-	-
10	MEZ (10 ng/ml)	1+	-
	IFN- $\beta$ (2000 U/ml)	3+	-
15	IFN- $\gamma$ (2000 U/ml)	2+	-
20	RA + MEZ (2.5 $\mu$ M + 10 ng/ml)	1+	-
	MPA + MEZ (3.0 $\mu$ M + 10 ng/ml)	3+	-
25	IFN- $\beta$ + IFN- $\gamma$ (1000 U/ml + 1000 U/ml)	4+	-
30	IFN- $\beta$ + MEZ (2000 U/ml + 10 ng/ml)	4+ <sup>g</sup>	+
35	-----		
40	*H0-1 cells were grown for 96 hr or for 6 or 7 days (with medium changes after 3 or 4 days) in the presence of the agents indicated. For morphology, cells grown for 96 hr in the test agent were observed microscopically. For melanin synthesis, results are for 6 day assays for RA and MPA (51) or 7 day assays for MEZ, IFN- $\beta$ , IFN- $\gamma$ , IFN- $\beta$ + IFN- $\gamma$ and IFN- $\beta$ + MEZ (10,50). For tyrosinase assays, results are for 6 day assays for RA, MPA and MEZ (51). Growth suppression (reversible and terminal cell differentiation) assays, refer to cultures treated with the indicated compound(s) for 96 hr prior to cell number determination, or treated for 96 hr and then grown for 2 weeks (with medium changes every 4 days) in the absence of compound prior to cell number determination.		
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-137-

<sup>b</sup>Morphology changes refer to the development of dendrite-like processes 96 hr after growth in the indicated compound. + = presence of dendrite-like processes; - = no dendrite-like processes.

<sup>c</sup>Melanin assays were determined as described in refs. 10,50,51. Results are expressed as relative increases based on separate data presented in refs. 10,50,51. N.T. = not tested.

<sup>d</sup>Tyrosinase assays were performed as described in ref. 11. Relative increases (of a similar magnitude) were found for RA, MPA and MEZ after 6 days exposure to these agents (51). N.T. = not tested.

<sup>e</sup>Reversible growth suppression indicates resumption of cell growth after treatment with the indicated compound(s) for 96 hr, removal of the test agent and growth for 14 days in compound(s) free medium. Further details can be found in ref. 14. The degree of initial 96 hr growth suppression is indicated as: - = no significant change in growth (< 10% reduction in growth in comparison with untreated control cultures); 1+ = ~30% reduction in growth in comparison with untreated control cultures; 2+ = ~40% reduction in growth in comparison with untreated control cultures; 3+ = ~50 to 60% reduction in growth in comparison with untreated control cultures; 4+ = ~80% reduction in growth in comparison with untreated control cultures.

<sup>f</sup>The combination of IFN- $\beta$  + MEZ results in irreversible growth suppression.

<sup>g</sup>Terminal cell differentiation indicates the loss of proliferative capacity after treatment with the indicated compound(s) for 96 hr, removal of the test agent and growth for 14 days in compound(s) free medium. Further details can be found in (14).

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The studies briefly described above indicate that changes in growth, morphology, melanin synthesis and tyrosinase activity can be dissociated from the induction of terminal differentiation in H0-1 melanoma cells. However, the irreversible loss of proliferative capacity and terminal differentiation resulting from treatment with IFN- $\beta$  plus MEZ appear to be correlated phenomena. Employing the various agents described above it will be

-138-

possible to determine which gene expression changes are related to the various components of the differentiation process in human melanoma cells.

5 Monoclonal antibodies (MAbs) have recently been developed which recognize a series of hnRNP proteins, designated P2Ps, which display a marked reduction in both 3T3T cells and human keratinocytes induced to terminally  
10 cells that have retained the ability to traverse the cell cycle, including cells reversibly growth arrested. A loss of P2Ps is also observed in cells that have irreversibly lost proliferative potential as a consequence of senescence, as well as induction of  
15 terminal differentiation (52,53). In contrast, 3T3T cells transformed by SV40 do not undergo the terminal step of differentiation and these cells also do not show a suppression of P2P expression (52). These results support the concept that P2Ps may be directly linked to  
20 proliferative capacity of cells and may prove useful as a general marker for terminal cell differentiation. In collaboration with Dr. Robert E. Scott (University of Tennessee Medical Center, Memphis, TN) applicants have begun to determine the level of P2Ps in human melanoma  
25 cells induced to terminally differentiate by exposure to IFN- $\beta$  plus MEZ and MPA plus MEZ (Fig. 14). When induced to terminally differentiate, a reduction in P2Ps was apparent in several independent human melanoma cell lines (data from F0-1 human melanoma cells is shown in Fig.  
30 14).. In contrast, employing human melanocytes immortalized by the SV40 T-antigen gene (54), IFN- $\beta$  plus MEZ did not induce terminal differentiation or a reduction in P2Ps, whereas MPA plus MEZ resulted in a loss of proliferative capacity and a reduction in P2Ps.  
35 In both F0-1 and FM516 SV cells, treatment with IFN- $\beta$ ,

-139-

MEZ or MPA alone did not reduce P2Ps even though growth was suppressed (data not shown). Induction of terminal differentiation by IFN- $\beta$  + MEZ in HO-1 cells resulted in a loss of P2Ps, whereas either agent employed alone did not reduce P2P levels (data not shown). Although these results are preliminary, they suggest that the chemical induction of terminal differentiation and the irreversible loss of proliferative capacity in human melanoma cells is associated with a reduction in P2Ps.

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B. Gene Expression Changes induced in Human Melanoma Cells Displaying Reversible Growth Suppression, Reversible Commitment to Differentiation and Terminal Cell Differentiation.

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Applicants have begun to determine the spectrum of gene expression changes associated with growth suppression, morphologic alterations, increased melanin synthesis, enhanced tyrosinase activity and/or induction of terminal differentiation in human melanoma cells (12-14). The agents applicants have chosen result in reversible growth suppression, induction of melanin synthesis, morphologic alterations, enhanced tyrosinase activity, induction of a reversible commitment to differentiation or terminal differentiation with a concomitant loss of proliferative capacity in HO-1 melanoma cells (Table 2). The genes applicants have currently analyzed include: early response genes (*c-fos*, *c-myc*, *c-jun*, *jun-B*, *jun-D* and *gro/MGSA*) (14); interferon stimulated genes (*ISG-15*, *ISG-54*, *HLA Class I* and *HLA Class II*) (13, 14); cell adhesion molecules (*P-cadherin*, *E-cadherin*, *N-cadherin* and *N-CAM*) (14); extracellular matrix genes (*fibronectin* (*FIB*) and *tenascin*) (12, 14); cell surface proteoglycans/matrix receptors (*syndecan*,  $\beta_1$  *integrin*

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-140-

(major FIB receptor subunit),  $\alpha$ , integrin (major FIB receptor subunit)) (14); cytoskeleton genes (tropomyosin-1,  $\gamma$ -actin and  $\beta$ -actin) (14); and a housekeeping gene (GAPDH) (14). Using the gene probes indicated above, no unique gene expression change was found which only occurred in terminally differentiated H0-1 cells. These results indicate that commitment to differentiation and terminal differentiation in H0-1 melanoma cells is associated with specific patterns of overlapping gene expression changes. As will be discussed below, an interesting change in gene expression observed in both H0-1 cells committed to differentiate and induced to terminally differentiate was the induction and enhanced expression of type I interferon responsive genes and the gro/MGSA gene. These results have led to the hypothesis that specific autocrine feedback loops may contribute or are associated with the differentiation process in human melanoma cells.

20 C. Changes in Cell Cycle and Early Immediate Response Genes During the Induction of Terminal Differentiation in Human Melanoma Cells.

As discussed above, treatment of H0-1 cells with the combination of IFN- $\beta$  + MEZ results in growth suppression that is apparent by 24 hr following exposure to these inducing agents (Fig. 12) (10, 14). This system has been used to evaluate the effects of the different inducers, alone and in combination, on the expression of cell cycle regulated genes, including cdc2, cyclin A, cyclin B, histone 1, histone 4, proliferative cell nuclear antigen (PCNA), c-myc, p53 and Rb. These studies can be summarized as follows: (a) A reduction in cdc2 and histone 1 was apparent under all treatment conditions.

-141-

This effect was observed after 24 hr and was most dramatic in cells treated for 96 hr; (b) *c-myc* expression was marginally decreased by 24 hr treatment with MEZ and IFN- $\beta$  + MEZ, whereas significant suppression was observed by 96 hr especially in IFN- $\beta$  + MEZ treated H0-1 cells; (c) Both PCNA and p53 gene expression was reduced only in cells treated with IFN- $\beta$  + MEZ; and (d) Rb levels remained unchanged following any of the treatment protocols. In the case of *cdc2* and histone 1, IFN- $\beta$  + MEZ resulted in a decreased rate of transcription of these genes. Similarly, IFN- $\beta$  + MEZ decreased the stability of the *cdc2* and histone 1 mRNAs. Analysis of cell cycle distribution by FACS analysis indicated that both MEZ and IFN- $\beta$  + MEZ reduced the number of H0-1 cells undergoing DNA synthesis by 48-hour treatment. The most effective inhibitor of DNA synthesis in H0-1 cells was IFN- $\beta$  + MEZ. These results indicate that the induction of terminal differentiation in H0-1 cells by IFN- $\beta$  + MEZ is associated with a suppression in specific cell cycle related genes that occur at both a transcriptional and a postranscriptional level.

*c-fos*, *c-jun* and *jun-B* expression were superinduced in H0-1 cells treated with cycloheximide and IFN- $\beta$  + MEZ, indicating that these genes are immediate early response genes. Differences in the temporal kinetics of induction and the mechanism of enhanced expression were apparent between these early response genes in differentiation-inducer treated H0-1 cells (55). In the case of *c-fos*, IFN- $\beta$  + MEZ induced an increase in transcription of *c-fos* mRNA that was apparent after 1, 6 and 24 hr, but not after 96 hr treatment (55). In the case of *c-jun*, increased mRNA was apparent after 1, 6, 24 and 96-hour treatment with IFN- $\beta$  + MEZ. These changes in *c-jun* level did not involve increased transcription, but instead

-142-

resulted from an increase in half-life of the c-jun transcripts (55). In the case of jun-B, IFN- $\beta$  + MEZ increased both the transcription and steady-state levels of RNA after 1- and 24-hour treatment. High levels of jun-B mRNA were also apparent in H0-1 cells induced to terminally differentiate after treatment with IFN- $\beta$  + MEZ. The continued increase in c-jun and jun-B mRNA levels in terminally differentiated H0-1 cells suggests that these genes may contribute to maintenance of the terminal differentiation phenotype (55).

D. Autocrine Loops Induced in Human Melanoma Cells Treated with IFN- $\beta$  plus MEZ.

As indicated above, continuous treatment with IFN- $\beta$  + MEZ for 96-hour results in terminal differentiation in H0-1 human melanoma cells. This process correlates with specific patterns of gene expression changes, including the induction of two interferon stimulated genes, ISG-15 and ISG-54, and melanocyte growth stimulatory activity (gro/MGSA) (14). These observations suggested the possibility that induction of a reversible commitment to differentiation and terminal differentiation might be associated with the production of differentiation promoting factors (DPFs) (possibly including IFN- $\beta$  or an IFN- $\beta$ -like cytokine and melanoma growth stimulatory activity (gro/MGSA)). These DPFs could then induce by an autocrine mechanism the transcription and steady-state mRNA expression of interferon stimulated genes (ISG) and the gro/MGSA gene in H0-1 cells reversibly committed to differentiation or terminally differentiated. To further explore the relationship between treatment time and induction of differentiation and to test the autocrine hypothesis, applicants performed two types of experiments. In the first set of studies (In-Out), H0-1

-143-

cells were treated with various agents (including IFN- $\beta$  + MEZ, MPA + MEZ, RA + MEZ and MEZ (at a high dose of 50 ng/ml) for 24 hr, cells were washed 2X with DMEM without FBS, DMEM containing 10% FBS was added to cultures and  
5 total cytoplasmic RNA was isolated 72 hours later. For the second set of experiments (Conditioned-Medium), cells were processed as indicated above for In-Out experiments except after 72 hour growth in the absence of inducer, medium was collected (and contaminating cells were  
10 removed by centrifugation).

Using the In-Out and Conditioned-Medium protocols it was demonstrated that: (a) At the doses employed, IFN- $\beta$  + MEZ is a more potent inducer of gene expression changes and  
15 the only combination capable of inducing terminal differentiation in H0-1 cells; and (b) Conditioned medium from H0-1 cells reversibly committed to differentiate or terminally differentiated by IFN- $\beta$  + MEZ induce specific programs of gene expression changes in H0-1 cells that  
20 are similar to those induced directly by the inducing agents (14). In addition, conditioned medium from IFN- $\beta$  + MEZ treated H0-1 cells also induces morphologic changes and suppresses growth when added to H0-1 or F0-1 human melanoma cells (data not shown). These results support  
25 the hypothesis that induction of terminal differentiation in H0-1 melanoma cells is associated with specific changes in gene expression, some of which may be mediated by or associated with an autocrine feedback mechanism.

30 E. Cloning of Melanoma Differentiation Associated (mda) Genes Induced in H0-1 Melanoma Cells treated with IFN- $\beta$  plus MEZ.

The ability to identify and isolate differentially  
35 expressed genes between two similar or different cell

-144-

types is now readily achievable using subtraction hybridization (rev. 57, 58). A procedure for constructing subtracted libraries have been developed that is both sensitive and efficient (49). The application of this approach for the identification of genes differentially expressed in H0-1 cells treated with IFN- $\beta$  plus MEZ is outlined in Figure 8. Tester and driver cDNA libraries are directionally cloned into the commercially available  $\lambda$  Uni-ZAP phage vector. Subtraction hybridization is then performed between double-stranded tester DNA and single-stranded driver DNA prepared by mass excision of the libraries. The subtracted cDNAs are efficiently cloned into the  $\lambda$  Uni-ZAP phage vector that can be easily managed for both screening and gene characterization. The applicability of the procedure was demonstrated by the identification of cDNAs displaying enhanced expression in human melanoma cells, H0-1, induced to terminally differentiate by treatment with IFN- $\beta$  + MEZ (Fig. 10). A single round of subtraction of untreated H0-1 control (*Ind*) cDNAs from IFN- $\beta$  + MEZ treated (*Ind*<sup>+</sup>) cDNAs generated a series of cDNAs displaying differential expression in untreated versus differentiation inducer-treated H0-1 cells, termed melanoma differentiation associated (*mda*) cDNAs. Employing the approach briefly described above, a total of 23 differentially expressed *mda* cDNAs have been isolated which represents only a portion of the subtracted H0-1 IFN- $\beta$  + MEZ cDNA library. Partial sequence analysis of these 23 *mda* genes resulted in the identification of known genes, including a human TPA-inducible gene, the human apoferritin H gene, the IFP-53 (gamma-2 protein) gene, the IL-8 (monocyte-derived chemotactic factor) gene, the vimentin gene, the hnRNP A2 protein gene, human macrophage inflammatory protein



-145-

(GOS19-1) and the IFN- $\beta$ -inducible gene ISG-56. In addition, 6 cDNAs have been identified which do not have sequences previously reported in any of the gene data bases. As predicted based on the subtraction protocol employed, some of the *mda* genes are induced within 24 hours by: IFN- $\beta$  and IFN- $\beta$  + MEZ (e.g., *mda*-1 and *mda*-2); MEZ and IFN- $\beta$  + MEZ (e.g., *mda*-3); IFN- $\beta$ , MEZ and IFN- $\beta$  + MEZ (e.g., *mda*-4); and only by IFN- $\beta$  + MEZ (e.g., *mda*-5 and *mda*-6) (Fig. 10). A potentially important group of *mda* genes is represented by cDNAs displaying significantly enhanced expression in H0-1 cells treated with IFN- $\beta$  + MEZ for 96 hours and displaying terminal cell differentiation, i.e., *mda*-5, *mda*-6, *mda*-7 and *mda*-9 (all representing novel genes) (Fig. 15). Additional *mda* cDNAs which may prove of value in understanding growth control in human melanoma cells have been identified which are expressed in both terminally differentiated H0-1 cells and H0-1 cells induced to undergo a reversible suppression in growth by treatment with IFN- $\beta$  + IFN- $\gamma$ , i.e., *mda*-4, *mda*-5, *mda*-7 and *mda*-8 (Fig. 15). Increased expression of a number of *mda* genes following treatment with IFN- $\beta$  + MEZ are not restricted to H0-1 cells, since increased *mda* gene expression is also induced in additional human melanomas induced to terminally differentiate by treatment with IFN- $\beta$  + MEZ (data not shown). The studies described above indicate the feasibility of using subtraction hybridization to identify genes that may directly mediate or represent markers of terminal differentiation in human melanoma cells.

F. Gene Expression Changes induced in the C8161 Melanoma Cells and Chromosome 6 Microcell C8161 Hybrids.

-146-

Recent studies by Welch et al. (42) indicate that insertion of a normal chromosome 6 (by the microcell chromosome replacement technique) into the C8161 human melanoma cell line results in a suppression of metastatic, but not tumorigenic potential in nude mice. Treatment of C8161 cells for 4 or 7 days with IFN- $\beta$  + MEZ (1000 units/ml + 10 ng/ml) results in terminal cell differentiation. In contrast, under similar conditions, C8161 cells containing chromosome 6 (Clone 6.1, 6.2 and 6.3) display morphological changes and growth suppression but cells retain proliferative potential, i.e., the combination of agents induces a reversible commitment to differentiation as opposed to terminal differentiation. A lack of terminal differentiation in 6.1, 6.2 and 6.3 cells was demonstrated by removing the test agents and growth in inducer free medium (data not shown). Analysis of gene expression in parental C8161 and 6.1, 6.2 and 6.3 cells indicated differences that correlated with the presence of a normal chromosome 6. Specific differences in gene expression after 4 days incubation with IFN- $\beta$  and MEZ, alone and in combination, include: (a) induction of IL-8 mRNA (which was identified as an *mda* cDNA in H0-1 cells treated with IFN- $\beta$  + MEZ) in MEZ and IFN- $\beta$  + MEZ treated C8161 cells, but not in 6.1, 6.2 or 6.3 cells; (b) induction of HLA Class I antigen mRNA by IFN- $\beta$ , MEZ and IFN- $\beta$  + MEZ in C8161, but only by IFN- $\beta$  and IFN- $\beta$  + MEZ in 6.1, 6.2 and 6.3 cells; and (c) reduced induction of ISG-15 expression in C8161 cells versus 6.1, 6.2 and 6.3 cells treated with IFN- $\beta$  and IFN- $\beta$  + MEZ. The studies briefly described above indicate that IFN- $\beta$  + MEZ is more effective in inducing terminal differentiation in the less differentiated metastatic C8161 melanoma cells than the more differentiated 6.1, 6.2 and 6.3 cells. This model system should prove useful in determining the role of specific *mda* genes in expression of the

-147-

tumorigenic and metastatic phenotype by human melanoma cells.

#### DESIGN AND METHODS

5

A. Specific Aim #1: Determine the pattern and regulation of expression of the melanoma differentiation associated (*mda*) genes in melanocytes, nevi, radial growth phase melanoma, vertical growth phase melanoma and  
10 metastatic melanoma cells.

##### 1. Rationale and General Approach:

Applicants have tested the hypothesis that human melanoma  
15 cells display aberrant patterns of differentiation and by appropriate chemical treatment they can be induced to undergo an irreversible loss in proliferative capacity without a loss of viability, i.e., terminal cell differentiation (10,11,14). Using the combination of  
20 IFN- $\beta$  + MEZ applicants have demonstrated that the reprogramming of human melanoma cells to terminally differentiate can be achieved in vitro (10,11,14). On the basis of a second hypothesis, i.e., terminal differentiation is associated with the selective  
25 activation of specific programs of gene expression, applicants have developed and used a modified subtraction hybridization protocol to identify genes displaying enhanced expression under conditions resulting in terminal cell differentiation (49). These studies have  
30 resulted in the cloning of a series of genes, termed melanoma differentiation associated (*mda*) genes, which display such specificity. The purpose of the studies to be described below are to: (a) characterize the *mda* genes with respect to their level of regulation in H0-1  
35 melanoma cells, i.e., transcriptional versus

-148-

posttranscriptional mechanisms of induction; (b) determine if the expression of specific *mda* genes correlate with a defined stage in melanoma development; (c) continue screening our subtracted IFN- $\beta$  + MEZ cDNA library to identify additional *mda* genes which display enhanced expression in growth arrested and terminally differentiated human melanoma cells; and (d) use additional subtraction steps to enrich for genes only expressed at high levels in H0-1 cells induced to terminally differentiate.

(a) Defining the level of regulation of *mda* genes in H0-1 cells treated with IFN- $\beta$  + MEZ: Initial studies will focus on the mechanism by which IFN- $\beta$  + MEZ increases the expression of cloned *mda* genes that are significantly upregulated (4- to >20-fold) by this combination of inducing agents in H0-1 cells. The genes to be analyzed will include *mda*-5, *mda*-6, *mda*-7, *mda*-8 and *mda*-9 described in Preliminary Studies, which represent novel IFN- $\beta$  + MEZ-inducible genes not previously reported in the Gene Bank or the EMBL gene data base. The order of experiments will include: (1) determining the temporal kinetics of induction of the *mda* genes; (2) determining if the level of induction of specific *mda* genes occurs at a transcriptional level; (3) determining if any of the *mda* genes are immediate early (primary) response genes; and (4) determining if differentiation results in an altered stability of the *mda* transcripts.

(i) The screening strategy used to identify the *mda* genes involved Northern hybridization analysis of RNA isolated from H0-1 cells treated with IFN- $\beta$ , MEZ or IFN- $\beta$  + MEZ for 24 hr (49). Previous studies indicated that exposure to IFN- $\beta$  + MEZ for 24 hr resulted in a number of gene expression changes also observed in H0-1 cells induced to

-149-

terminally differentiate after 4 days exposure to this combination of agents (Preliminary Studies) (14). *mda* genes displaying increased expression in H0-1 cells after 24 hr treatment with IFN- $\beta$  + MEZ were subsequently evaluated for enhanced expression after 4 days treatment with the inducers. *mda*-5, *mda*-6, *mda*-7, *mda*-8 and *mda*-9 genes displayed enhanced expression in H0-1 cells treated with IFN- $\beta$  + MEZ for 24 or 96 hr. These results indicated that increased expression of the *mda* genes occurred within the first 24 hr of treatment and enhanced expression persisted during terminal cell differentiation. To determine if any of the *mda* genes becomes activated after a short exposure to the inducing agents, temporal kinetic studies will be performed. H0-1 cells will be treated for short-time periods (15, 30, 45, 60 and 120 min) with IFN- $\beta$ , MEZ and IFN- $\beta$  + MEZ, cytoplasmic RNA will be isolated, electrophoresed on 0.6% agarose gels, transferred to nylon filters and sequentially hybridized with the various *mda* genes and lastly with GAPDH (as a control for equal RNA levels under the various experimental conditions) (14). RNA from cells treated with the inducers will also be isolated every 2 hr over a 48 hr period to determine if any cell cycle kinetic changes occur in expression of the *mda* genes. An important question that also will be addressed is whether continued expression of any of the *mda* genes is required for maintenance of the terminal differentiation phenotype. This will be determined by analyzing RNA isolated from H0-1 cells treated with IFN- $\beta$  + MEZ: continuously for 7 days (cells are terminal, but still viable); for 4 days followed by incubation in growth medium without inducers for an additional 10 days (cells remain terminal); and after 10 days in cells treated for 24 hr followed by growth in inducer-free medium (cells regain proliferative potential, i.e., they

-150-

display a reversible commitment to terminal differentiation).

(ii) The studies described above will indicate if any of the *mda* genes is induced early after exposure to IFN- $\beta$  + MEZ. To determine if IFN- $\beta$  + MEZ induce expression of any of the *mda* genes by increasing their rates of transcription, nuclear run-on assays will be performed as described previously (55,58,59). Brief Description of Protocol: Nuclei will be isolated from H0-1 cells either untreated (control) or treated for 1, 6 and 24 hr with IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml) or IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml). RNA transcripts previously initiated by RNA polymerase II will be allowed to elongate in the presence of [ $^{32}$ P]UTP. Nuclear RNA will be isolated, purified by passing through a G-50 sephadex column followed by denaturing with 0.1M NaOH for 5 min on ice (55). Labeled nuclear RNA will be hybridized to nitrocellulose dot filters containing 2  $\mu$ g of plasmid DNA containing the *mda* genes, GAPDH DNA or pBR322 DNA (negative control) which has been denatured by boiling in 0.1M NaOH for 15 min followed by dilution with cold 2M NaCl (55).

(iii) *c-fos*, *c-jun* and *jun-B* are immediate early (primary) response genes, i.e., induction is not dependent on new protein synthesis, but rather utilizes existing transcription factors, in IFN- $\beta$  + MEZ treated H0-1 cells (55). To determine if any of the *mda* genes is an immediate early (primary) response gene, experiments using the protein synthesis inhibitor cycloheximide will be performed (55). Brief Description of Protocol: Approximately  $2 \times 10^6$  H0-1 cells will be untreated or treated with IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml) or

-151-

IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml) for 1 hr in the absence and presence of 50  $\mu$ g/ml cycloheximide (added 15 min prior to any other additions) (55). Total RNA will be isolated, electrophoresed in 0.6 % agarose, transferred to nitrocellulose filters and hybridized sequentially with the *mda* genes and lastly with GAPDH (14, 49, 60). By definition, if any of the *mda* genes are immediate early response genes they will be induced within 1 hour by IFN- $\beta$  + MEZ in both the absence and presence of cycloheximide. Another indication that the *mda* genes are primary response genes would be the phenomenon of superinduction, i.e., the massive over-accumulation of immediate early response transcripts which occur when cells are treated simultaneously with an inducer and protein synthesis inhibitors (55,61,62).

(iv) The ability of IFN- $\beta$  + MEZ to increase c-jun expression does not involve an increase in transcription, but rather results from an increase in the stability of c-jun mRNA (55). To determine if IFN- $\beta$  + MEZ alters the expression of specific *mda* genes by a posttranscriptional mechanism, studies using the transcription inhibitor actinomycin D will be performed (55). Brief Description of Protocol: Approximately  $2 \times 10^6$  H0-1 cells will be untreated or treated with IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml) and IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml) for 24 hr followed by no addition or the addition of actinomycin D (5  $\mu$ g/ml) for 30 min, 1 hr, 2 hr and 3 hr prior to RNA isolation (55). The RNAs will be analyzed by Northern hybridization and probing with the different *mda* genes or GAPDH (14,49,55). Radioautograms will be scanned using a densitometer to quantitate cellular RNA levels (55). These studies will indicate if IFN- $\beta$  + MEZ can alter the stability, i.e., the half-life, of any of the *mda* gene

-152-

transcripts.

Summary: These studies will indicate if *mda-5*, *mda-6*,  
*mda-7*, *mda-8* or *mda-9* are primary response genes and if  
5 their enhanced expression in human melanoma cells treated  
with IFN- $\beta$  + MEZ results from a transcriptional and/or a  
posttranscriptional mechanism.

(b) Analysis of *mda* gene expression during the process of  
10 melanoma development: A basic tenet of our terminal  
differentiation hypothesis is that the *mda* genes may  
represent genes normally expressed or expressed at higher  
levels in melanocytes and/or in the early stages of  
melanoma development, i.e., nevi, early radial growth  
15 phase (RGP) primary melanoma and/or early vertical growth  
phase (VGP) primary melanoma. If this concept is  
correct, then a prediction would be that specific *mda*  
genes would display reduced expression in late VGP  
melanoma and metastatic melanoma versus melanocytes, nevi  
20 and early stage melanomas. Support for this hypothesis  
comes from preliminary studies indicating that  
SV40-transformed human melanocytes express high levels of  
*mda-5*, *mda-6* and *mda-7* mRNA in the absence and in the  
presence of IFN- $\beta$  + MEZ (data not shown). To directly  
25 test this hypothesis applicants will analyze RNA obtained  
from cell cultures of melanocytes, dysplastic nevi (DN  
91D, DN (MM92E)), RGP melanoma (WM 35), early VGP  
melanoma (WM 793, WM902b), advanced VGP melanoma (WM  
983a, WM 115) and metastatic melanomas (WM 9, MeWo,  
30 SK-MEL 28, WM 239) (1,16,21,22,27) (to be supplied by Dr.  
Meenhard Herlyn). Applicants realize that cell cultures  
may not always reflect processes occurring in vivo,  
however, cell cultures will provide an initial indication  
of which *mda* genes to emphasize using in situ  
35 hybridization approaches with clinical specimens of



-153-

melanocytes, pre-malignant skin lesions, primary melanoma and metastatic melanoma. The procedures to be used for in situ hybridization with oligonucleotide probes will be as described by Reed et al. (63) and Biroc et al. (64) (to be performed in collaboration with Dr. Anthony P. Albino, Memorial Sloan-Kettering Cancer Center, New York). In the studies by Reed et al. (63) in situ hybridization with a basic fibroblast growth factor (bFGF) oligonucleotide has been successfully used to determine differential expression of bFGF in melanocytic lineage tissue specimens. As an additional approach for determining *mda* gene expression in clinical specimens, RNA isolated directly from patient samples displaying different stages of melanoma evolution (to be supplied by Dr. Herlyn) will be evaluated by Northern analysis (14,49) and where necessary to increase sensitivity of detection by RT-PCR analysis (65) for expression of the *mda* genes. In previous studies analyzing expression of the epidermal growth factor receptor, a wide spectrum of human central nervous system tumors obtained from patients was evaluated (66). This study clearly indicated that intact and high-quality RNA could be obtained efficiently from patient material and utilized for comparative gene expression studies.

As indicated in Background and Significance, metastatic melanoma cells are often inhibited in their growth by TPA (or MEZ), whereas the in vitro growth of normal melanocytes and nevi are stimulated by TPA (or MEZ) (1,15,16,20-23). Similarly, many metastatic melanoma cell lines are growth inhibited by IFN- $\beta$  (8,13-15), whereas under optimal growth conditions normal melanocytes are not growth inhibited by IFN- $\beta$  (even though TPA is incorporated in the growth medium) (15). These results indicate that progression from melanocyte

-154-

to malignant melanoma involves a change in responsiveness to both TPA (or MEZ) and IFN- $\beta$ . Based on these observations, it would be predicted that a stage-specific effect will be observed in melanocyte lineage cells exposed to the combination of IFN- $\beta$  + MEZ. If this effect is observed, it will provide a direct test of the potential involvement of the *mda* genes in the process of growth inhibition and terminal differentiation resulting from treatment with IFN- $\beta$  + MEZ. The melanocyte lineage cell lines, i.e., melanocyte, dysplastic nevus, RGP primary melanoma, early VGP primary melanoma, advanced VGP melanoma and metastatic melanoma (supplied by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA), will be used to directly determine: (1) if the combination of IFN- $\beta$  + MEZ displays a stage-specific growth inhibitory effect and the induction of terminal differentiation; and (2) if the induction of growth suppression and/or the induction of terminal differentiation in stage-specific melanocyte lineage cells correlates with changes in the expression of specific *mda* genes. These studies will be conducted as described previously (10,14). Brief Description of Protocols: For growth studies: cells will be seeded at 2.5 or 5 X 10<sup>4</sup> cells/35 mm plate; 24 hr later the medium will be changed with no additions (Control), 1000 and 2000 units/ml of IFN- $\beta$ , 1, 10 and 50 ng/ml MEZ and combinations of IFN- $\beta$  + MEZ; cell numbers will be determined daily (with a medium change at day 4) over a seven day period. For reversibility studies, cells will be treated for 24 hr or 4 days with the different inducers followed by growth in inducer free medium for an additional 7 to 14 days at which time cell numbers will be determined. RNA will be isolated from cells treated for 24 hr, 4 days and 7 days and analyzed by Northern

-155-

blotting hybridization for expression of the *mda* genes as described previously (14). Biochemical markers for growth suppression and differentiation will include: an analysis of P2P levels using appropriate monoclonal antibodies and Western blotting analysis (Preliminary Studies) (52,53); antigenic markers, such as the GD3 ganglioside and Class II MHC, and fluorescence activated cell sorter analysis as described previously (50,67,68); and a determination of melanin levels as described previously (69).

Studies designed to identify stage-specific effects of IFN- $\beta$  + MEZ and the role of the *mda* genes in growth suppression and terminal cell differentiation in melanocyte lineage cells will be aided by using three recently described model systems. These will include: (A) the transformed human melanocyte cell lines 10Wras/early and 10Wras/late (supplied by Dr. Anthony P. Albino, Memorial-Sloan Kettering Cancer Center, NY, NY) (20); (B) the metastatic human melanoma cell line C8161 and the tumorigenic but non-metastatic C8161 clones containing a normal human chromosome 6 (supplied by Dr. Dan Welch, Milton S. Hershey Medical Center, Hershey, PA) (42,43); and (C) RGP or early VGP primary human melanomas (WM 35, WM 1341B and WM 793) which have been selected by injection with matrigel in nude mice for a more progressed tumorigenic and metastatic phenotype (e.g., 35-P1-N1, 35-P1-N2, 35-P1-N3, 1341-P1-N1, 1341-P1-N2, 1341-P2-N1 etc. (cell line: passage number; nude mouse number)) (supplied by Dr. Robert S. Kerbel, Sunnybrook Medical Center, Toronto, Canada) (25,70). The 10Wras/early and 10Wras/late cells are human melanocytes transformed by a retrovirus containing the viral Ha-ras oncogene which display specific traits associated with melanoma progression (20). 10Wras/early cells display

-156-

TPA dependence, are nontumorigenic in nude mice, and express many of the antigenic markers present in normal melanocytes (20). In contrast, the 10Wras/late cells are inhibited by TPA, tumorigenic in nude mice, contain many of the cytogenetic changes seen in metastatic melanoma (including modifications in chromosome 1, 6 and 9) and express many of the same growth factor genes as metastatic human melanoma (20). As discussed in Preliminary Studies, applicants have begun to analyze gene expression changes in IFN- $\beta$  + MEZ treated C8161 cells and C8161 cells containing a microcell-transferred normal chromosome 6 (6.1, 6.2 and 6.3). Unlike C8161 cells, 6.1, 6.2 and 6.3 cells are not metastatic in nude mice (42) and they are not induced to terminally differentiate when treated with the same concentration of IFN- $\beta$  + MEZ resulting in terminal differentiation in C8161 cells. 6.1, 6.2 and 6.3, therefore, may represent human melanoma cells which have been reverted to a less advanced stage in melanoma development. As discussed previously, since surgical removal of RGP or early stage VGP primary human melanomas results in a cure of this disease, it has not previously been possible to analyze more aggressive variants derived from the same early stage melanomas. Dr. Kerbel and colleagues (25) have potentially overcome this problem by injecting RGP and early VGP primary human melanomas in combination with matrigel into nude mice. Tumors which then developed were found to be tumorigenic in nude mice without the requirement for matrigel and they also acquired a "cytokine resistance phenotype" which is associated with melanoma progression (25,70). These three cell systems should prove extremely valuable in determining if *mda* gene expression correlates with specific stages of melanoma progression. To test this possibility applicants will conduct similar experiments as described

-157-

previously (4. A. 1. a) using these stage-specific cell lines. If our hypothesis suggesting that the response of melanocyte lineage cells to IFN- $\beta$  + MEZ is stage-specific is correct, then applicants would predict that the effect of these agents on growth, *mda* gene expression and terminal cell differentiation would be greater in 10Wras/late vs. 10Wras/early cells, C8161 cells vs. chromosome 6 containing C8161 clones and the more progressed vs. the less progressed RGP and early VGP primary melanoma cell lines.

Summary: These studies will indicate if a direct relationship exists between the state of progression of melanoma cells and *mda* gene expression. They will also indicate if the response to IFN- $\beta$  + MEZ induced growth suppression, *mda* gene expression and terminal cell differentiation is directly related to melanoma progression.

(c) and (d) Identification of additional *mda* genes which display enhanced expression in growth arrested and/or terminally differentiated human melanoma cells: As indicated in Preliminary Studies (E), only a small percentage (approximately 2.5%) of our subtracted IFN- $\beta$  + MEZ H0-1 library has been screened for differentially expressed genes associated with growth suppression and terminal differentiation in H0-1 cells. It is therefore conceivable that a number of additional *mda* genes, which still remain to be identified, are present in the subtracted IFN- $\beta$  + MEZ H0-1 library. The initial subtraction hybridization approach applicants have used has resulted in the identification of cDNAs displaying enhanced expression in H0-1 cells treated with: IFN- $\beta$  and IFN- $\beta$  + MEZ; MEZ and IFN- $\beta$  + MEZ; IFN- $\beta$ , MEZ and IFN- $\beta$  + MEZ; and only IFN- $\beta$  + MEZ (49). To identify additional

-158-

*mda* genes which are preferentially expressed at elevated levels in cells induced to terminally differentiate following treatment with IFN- $\beta$  + MEZ applicants will perform additional subtraction hybridization steps.

5 Brief Description of Protocol: cDNA libraries will be constructed from H0-1 cells treated with IFN- $\beta$  (2000 units/ml) or MEZ (10 ng/ml) for 2, 4, 8, 12 and 24 hr as previously described (49,57). The IFN- $\beta$  and MEZ cDNA libraries will be converted into single-stranded DNA

10 which will then be biotinylated using photoactivatable biotin and used as the Driver DNA as described previously (49). The H0-1 IFN- $\beta$  + MEZ (*Ind*<sup>+</sup>) subtracted cDNA library will be converted into double stranded DNA and the double-stranded inserts will be isolated and used as

15 the Tester DNA (49). The Driver DNA will then be subtracted away from the Tester DNA resulting in an enriched H0-1 IFN- $\beta$  + MEZ (Enriched-*Ind*<sup>+</sup>) subtracted cDNA library (49). As an alternate approach to specifically identify genes displaying increased elevation in

20 terminally differentiated melanoma cells, cDNA libraries will be constructed from H0-1 cells treated for 4 or 7 days with IFN- $\beta$ , MEZ and IFN- $\beta$  + MEZ. Subtraction hybridization will then be conducted as described (49) using these libraries to identify cDNAs only expressed at

25 increased levels in IFN- $\beta$  + MEZ-treated terminally differentiated H0-1 cells.

Summary: Additional screening of our current H0-1 IFN- $\beta$  + MEZ (*Ind*<sup>+</sup>) offers the potential of identifying more

30 differentially expressed *mda* genes. By constructing additional cDNA libraries from H0-1 cells treated singularly with IFN- $\beta$  or MEZ and subtracting this information away from cDNA libraries prepared from H0-1 cells treated with IFN- $\beta$  + MEZ, the identification of

-159-

additional *mda* genes displaying enhanced expression specifically in terminally differentiated melanoma cells should result.

- 5 B. Specific Aim #2: Analyze the relationship between *mda* gene expression and the induction of reversible commitment to differentiation, growth suppression without the induction of differentiation, DNA damage and stress responses and induction of terminal differentiation in  
10 human melanoma and other model differentiation systems.

1. Rationale and General Approach:

15 Induction of terminal differentiation in human melanoma cells, as well as other cell types such as myoblasts, neuroblastoma and leukemic cells, is associated with an irreversible loss in proliferative ability (rev 13,71). It is therefore reasonable to assume that some of the *mda* genes applicants have identified may also display  
20 enhanced expression in growth arrested melanoma (and other cell types) or melanoma cells (and other cell types) treated with various DNA damaging and chemotherapeutic agents which also induce growth-related changes. Indeed, preliminary studies indicate that  
25 *mda-4*, *mda-5* and *mda-8* exhibit increased expression in terminally differentiated H0-1 cells as well as H0-1 cells induced to undergo reversible growth suppression by treatment with IFN- $\beta$  + IFN- $\gamma$  (50). In addition, *mda-4* also displays increased expression in H0-1 cells  
30 reversibly growth suppressed by caffeic acid phenethyl ester (72), vinblastine, tumor necrosis factor- $\alpha$  and X-irradiation. Additional *mda* genes have been identified which display enhanced expression only in H0-1 and other metastatic human melanoma cells treated with IFN- $\beta$  + MEZ  
35 (i.e., they appear to be melanoma specific) or in H0-1

-160-

and dissimilar cell types induced to lose proliferative capacity, including human breast and colon carcinoma (i.e., they appear to be growth and or differentiation specific and not melanoma specific). Based on these preliminary observations, it appears that specific *mda* genes may be restricted to melanoma lineage cells induced to lose growth potential and become terminally differentiated, while other *mda* genes may represent key genes involved in growth control processes in diverse cell types. It will, therefore, be important to determine whether changes in the expression of specific *mda* cDNAs are restricted to human melanoma cells induced to terminally differentiate or whether they also display modified expression during other programs of terminal differentiation, DNA damage and growth arrest. The studies described below are designed to determine: (a) the spectrum of cellular changes which induce enhanced *mda* gene expression in human melanoma and other cell types; and (b) if induction of growth suppression and terminal differentiation in other cell types results in the enhanced expression of specific *mda* genes.

(a) Analysis of *mda* gene expression in human melanoma (and other cell types) treated with growth suppressing agents, DNA damaging agents and chemotherapeutic agents: Since terminal differentiation in H0-1 cells is associated with an irreversible loss in proliferative capacity (10,11,14), the *mda* genes applicants have identified may represent genes involved in both cell growth and terminal cell differentiation. To explore the relationship between cell growth and terminal differentiation, studies will be conducted to determine the types of agents and treatment protocols which can induce *mda* gene expression in human melanoma and other human cell types. The agents and treatments to be tested



-161-

will include: growth suppression (incubation in reduced serum levels), heat shock, gamma irradiation, ultraviolet irradiation (UVA and UVB), carcinogenic and mutagenic agents (methyl methanesulfonate, ethyl methanesulfonate and 4-nitroquinoline-oxide), demethylating agents (5-azacytidine, phenyl butyrate), chemotherapeutic agents (vinblastine, vincristine, adriamycin, cis-platinum), tumor necrosis factor- $\alpha$ , protein synthesis inhibitors (cycloheximide, puromycin, anisomycin), DNA synthesis inhibitors (amphidicolin, hydroxylurea, ara-C), transcription inhibitors (actinomycin D), topoisomerase inhibitors (camptothecin), poly-ADP-ribose inhibitors (3-aminobenzamide), protein kinase C activators (TPA, teleocidin, synthetic PKC activators (ADMB and DHI) (73-75)) and phosphatase inhibitors (calyculin, okadaic acid). Initial studies will focus on H0-1 cells. Subsequent investigations will involve other melanoma cells (representing different stages of melanoma progression) and additional human cell types (normal fibroblast and epithelial cells, neuroblastoma, glioblastoma, carcinomas (prostate, breast and colon) and sarcomas). Brief Description of Protocol: H0-1 cells (or the other experimental cell type employed) will be treated with the various agents for different time periods (ranging from 1 hr to 24 hr; or with certain treatment protocols for 4 and 7 days) and with different doses of the test treatment or agent. RNA will be isolated, electrophoresed in 0.6 % agarose gels, transferred to nylon filters and hybridized sequentially with the different *mda* genes and lastly with GAPDH. If specific pathways appear to induce an *mda* gene then further biochemical studies will be conducted. For example, if PKC activators induce specific *mda* genes then studies will be performed using specific inactive analogs and inhibitors of PKC to determine the relationship

-162-

between PKC activation and induction of gene expression. Similarly, if a treatment protocol or agent is found to induce or enhance expression of an *mda* gene, then subsequent studies will be conducted to determine if this  
5 change in gene expression is transcriptional or post-transcriptional (see Specific Aim #1 for experimental details).

Summary: The studies briefly outlined above will indicate  
10 if *mda* gene expression can be induced in H0-1, other human melanoma cells and additional human cell types, by treatment with agents which can alter growth and/or differentiation. They will provide initial information relative to potential biochemical pathways which may  
15 mediate the induction or enhanced expression of the *mda* genes. These experiments will also identify which *mda* gene(s) to use in studies (described in C. Specific Aim #3.) designed to determine the potential functional significance of *mda* gene expression changes in the  
20 control of growth and differentiation in human melanoma cells and other human cell types.

(b) Analysis of *mda* gene expression during the process of terminal differentiation in human promyelocytic  
25 leukemia (HL-60) and human skeletal muscle cultures: Specific *mda* genes are expressed in human melanoma and additional human cell types undergoing growth suppression with and without the induction of terminal differentiation. To explore this phenomenon further and  
30 to determine if any of the *mda* genes are also expressed at elevated levels in additional differentiation model systems applicants will conduct experiments using the HL-60 promyelocytic leukemic cell line (76) and human  
skeletal muscle cells (77,78). TPA induces macrophage  
35 differentiation in HL-60 cells (79), whereas dimethyl

-163-

5 sulfoxide (DMSO) results in granulocytic differentiation in HL-60 cells (80). In addition, growth of HL-60 cells in medium containing DMSO for 5 days followed by growth in TPA results in cells which switch from a granulocytic to monocytic differentiation program (81). These studies indicate that specific monocytic or granulocytic lineages can be induced in HL-60 cells by appropriate chemical manipulation. By growing HL-60 cells in incremental increases of TPA and DMSO, applicants have isolated variant populations which display a quantitative resistance to either TPA- or DMSO-induced differentiation (76). These resistant variants do not, however, display cross-resistance to other inducers, i.e., TPA induces a similar pattern of differentiation in parental HL-60 and HL-60/DMSO<sup>R</sup> cells and DMSO induces a similar pattern of differentiation in parental HL-60 and HL-60/TPA<sup>R</sup> cells. Although IFN- $\alpha$ A and IFN- $\beta$  induce growth suppression in parental HL-60, TPA-resistant HL-60 (HL-60/TPA<sup>R</sup>) and DMSO-resistant HL-60 (HL-60/DMSO<sup>R</sup>) cells, they do not induce these cells to differentiate terminally (76). However, the combination of IFN- $\alpha$ A or IFN- $\beta$  and TPA results in a synergistic growth suppression and the induction of terminal differentiation in both parental and HL-60/TPA<sup>R</sup> cells (76). Similarly, the combination of IFN- $\alpha$ A or IFN- $\beta$  and DMSO results in synergistic growth suppression and the induction of terminal differentiation in both parental and HL-60/DMSO<sup>R</sup> cells (76). This experimental model will prove extremely valuable in determining if a correlation exists between enhanced *mda* gene expression and either growth suppression or growth suppression with the induction of specific programs of terminal differentiation in human myeloblastic leukemic cells. Brief Description of Protocols: Parental HL-60, HL-60/TPA<sup>R</sup> and HL-60/DMSO<sup>R</sup> cells will be incubated with

-164-

IFN- $\beta$  (2000 units/ml)  $\pm$  inducer (TPA at  $10^{-9}$  and  $10^{-6}$  M or DMSO 0.9 to 1.5%) for 1, 3 and 7 days (with fresh medium  $\pm$  additions added at day 4). Cell numbers and terminal differentiation (as monitored by the presence of morphologically mature cells and the ability of cells to reduce nitroblue tetrazolium (NBT) (granulocyte specific) or the production of nonspecific esterase (macrophage specific) will be determined (84). RNA will be isolated, electrophoresed in 0.6% agarose gels, transferred to nylon filters and hybridized sequentially with the different *mda* genes and lastly with GAPDH (14, 49). Since the induction of both growth suppression and terminal differentiation is concentration- and compound-dependent in parental HL-60 and variant HL-60 cells, the studies outlined above will indicate if a relationship exists between the degree of growth suppression and/or the induction of terminal differentiation and expression of the different *mda* genes in HL-60 cells.

Methods are available for the in vitro growth of myogenic muscle satellite cells obtained from normal adult human skeletal muscle (82). These cultures recapitulate normal myogenesis, a process that can be followed with specific morphologic and biochemical markers and thus provides a useful system for assessing the effects of various agents on the process of cellular differentiation in human cells (77, 78, 82). Using this model system, applicants have previously demonstrated that TPA (and related compounds) inhibit spontaneous and induced myogenesis, whereas IFN- $\alpha$ A enhances myogenesis (77, 78). Inhibition or enhancement of differentiation in human skeletal muscle cultures is associated with either the suppression or induction, respectively, of specific morphologic changes

-165-

(development of multinucleated myotubes) and changes in creatine kinase isoenzyme transition from CK-BB to the CK-MM form (77,78). Applicants have also developed SV40-immortalized human skeletal muscle cells which fail to undergo terminal differentiation when treated with IFN- $\alpha$ A (83). This experimental model will prove extremely valuable in determining if any of the *mda* genes are differentially expressed during the induction of terminal differentiation or growth suppression in human skeletal muscle cells. Brief Description of Protocols: Muscle cultures will be grown from human skeletal muscle biopsy specimens obtained from diagnostic evaluation (from patients of either sex and ranging from 6 months to 50 years of age) as previously described (77,78,82). Prior to myoblast fusion, the cells will be trypsinized and plated at  $2 \times 10^6$  cells/10 cm tissue culture plate, cultures will be incubated with IFN- $\beta$  (2000 units/ml) and MEZ (10 ng/ml), alone and in combination, for 1, 4 and 7 days (with an appropriate medium change at day 4). Cell numbers will be determined and differentiation will be monitored using morphologic (myoblast fusion) and biochemical (creatine kinase isoenzyme transition from CK-BB to CK-MM) criteria (77,78,82). RNA will be isolated, electrophoresed in 0.6 % agarose gels, transferred to nylon filters and hybridized sequentially with the different *mda* genes and lastly with GAPDH. When only small quantities of RNA are available, RT-PCR (65) will be used to determine expression of the appropriate *mda* gene in early passage human skeletal myoblast cultures. In this model system, IFN- $\beta$  promotes skeletal muscle differentiation whereas MEZ inhibits differentiation. By employing specific concentrations of IFN- $\beta$  + MEZ, it is possible to obtain either an enhancement in differentiation, no change in

-166-

differentiation or an inhibition in differentiation (78). This system should permit a direct determination of the relationship between *mda* gene expression and the induction of myogenesis in human skeletal muscle cultures.

Summary: The studies described briefly above will indicate if any of applicants *mda* genes display altered expression during the induction of growth suppression and terminal differentiation in human myeloid leukemic cells and human skeletal muscle cells. If changes are observed, subsequent studies could be conducted to determine if the *mda* genes display enhanced expression in other differentiation models, including the U937 human monoblastic leukemia cell which can be induced to undergo macrophage differentiation (81), the PC12 rat pheochromocytoma cell line which can be induced to undergo neuronal differentiation (85) and human neuroblastoma cells which can be induced to differentiate terminally when treated with retinoic acid or other agents. In addition, if *mda* gene expression changes are observed in HL-60 cells induced to terminally differentiate, further studies would be conducted using HL-60 cells and other inducing agents which result in growth arrest without differentiation, DNA damage and apoptosis and terminal cell differentiation (with and without apoptosis). These studies would indicate which cellular and biochemical changes in HL-60 cells result in induction of specific programs of *mda* gene expression.

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C. Specific Aim #3: Isolate full-length cDNAs of *mda* genes that may be involved in melanoma differentiation or progression and directly determine their potential functional role in the differentiation and progression of

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-167-

human melanoma.

1. Rationale and General Approach:

5 The ability to analyze the functional significance of specific *mda* genes will require the isolation of full-length cDNAs. Once a full-length cDNA has been identified for a specific *mda* gene it can be used to: (1) produce its encoded protein using an in vitro translation  
10 system; (2) generate polyclonal antibodies specific for peptide regions of the encoded protein; (3) determine the location of the *mda* gene product in human melanoma cells and in tissue sections from patients; (4) determine the effect of overexpression of the *mda* gene on induction of growth suppression and terminal differentiation; and (5)  
15 determine the effect of blocking *mda* gene expression (using antisense oligomers or expression vector constructs) on the ability of IFN- $\beta$  + MEZ to induce growth suppression and terminal cell differentiation.

20 The approaches to be utilized to identify full-length *mda* cDNAs, in vitro translate the full-length *mda* cDNAs, produce antibodies against specific peptides of the encoded proteins, determine the location of the encoded proteins and to construct and analyze the effect of sense and antisense oligomers and expression vector constructs  
25 on growth and differentiation in H0-1 and other cell types is described below.

(a) Strategy for isolating full-length *mda* cDNAs: Rapid  
30 amplification of cDNA ends (RACE) is a procedure for amplification of nucleic acid sequences from a mRNA template between a defined internal site and an unknown sequence representing either the 3' or 5' end of the mRNA (86-88). The RACE procedure will be used to obtain the  
35 complete sequence of the full-length *mda* cDNAs (5' ends)

-168-

using the sequences (already determined) as the templates. Two types of gene-specific primers will be synthesized: the RT primer for reverse transcription and the AMP primer for PCR amplification. The sequence of the AMP primer is located upstream of the RT primer. First strand cDNA synthesis is initiated from the RT primer. After first strand cDNA synthesis, the original mRNA template is destroyed with RNase H and unincorporated dNTPs and RT primers are separated from cDNA using Centricon spin filters (Amicon Corp.). An oligo-DA anchor sequence is then added to the 3' end of the cDNA using TdT (terminal d transferase) and dATP. PCR amplification is accomplished using the AMP primer and a mixture of oligo(dT)-adapter primer/adapter primer (1:9). The adapter primer contains a specific sequence which, in the form of dsDNA, can be recognized and digested by the restriction enzymes SalI and XhoI. Following amplification, the RACE products will be digested with specific restriction enzymes (which do not cut the cDNA internally) and cloned into an appropriate plasmid (such as pBlueScript). The clones with specific inserts will be selected by screening (using DNA filter hybridization) and multiple independent clones of each gene will be used simultaneously for DNA sequencing to eliminate possible errors in sequence determination as a result of misincorporations occurring during the PCR amplification process.

(b) Characterization of full-length *mda* cDNAs: Full-length *mda* cDNAs will be used to obtain information about the *mda*-encoded proteins and the potential function of these genetic elements in regulating growth and differentiation of human melanoma cells. As will be described below, in vitro translation will be used to obtain the *mda*-encoded proteins. Once the protein



-169-

structure is determined, synthetic peptides will be constructed and used to generate antibodies specific for defined regions of the *mda* proteins. Antibodies will be used to determine the location of the *mda* proteins in melanoma and other cell types and tissue sections.

(i) Determination of protein structure and development of polyclonal antibodies specific for *mda* genes: Once full-length *mda* cDNAs have been isolated and sequenced, information will be available relative to the presence of open reading frames and the amino acid composition of the putative proteins encoded by these *mda* genes. To directly determine the size of the proteins encoded by the *mda* genes, full-length cDNAs will be subcloned into the pGEM-1 vector and transcribed in vitro using SP6 polymerase (Promega Corp., Madison, WI) as described (89). The in vitro transcribed RNA will be translated in a rabbit reticulocyte lysate (Amersham) in the presence of [<sup>35</sup>S]methionine according to the manufacturer's instructions and dialyzed against 10 mM Hepes, pH 7.9, 1 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 20% glycerol, 100 mM NaCl, 100 μM ZnCl<sub>2</sub> at 4°C overnight. Protein products will be analyzed by electrophoresis in an SDS/10-20% polyacrylamide gradient gel. Based on predicted amino acid structure of the protein, i.e., hydrophobicity value, antigenicity value and turn structure based on the deduced sequences of the *mda* cDNA, specific amino acids will be chosen for generating synthetic peptides (75,90). Synthetic peptides will then be used to generate polyclonal antibodies (Hazelton Laboratories, Denver, PA or Cappel Laboratories, Durham, NC). Brief Description of Protocol: The synthetic peptides will be conjugated with carrier proteins, bovine serum albumin and CGG (chicken γ-globulin) as described (90). Two and one-half

-170-

mg of peptide and 5 mg of carrier protein in double-distilled water will be incubated with 20 mg of either ethyl CDI (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride) (Sigma) or morpho CDI (1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate) (Sigma) in water at room temperature for 2 hours and dialyzed against PBS, pH 7.2. Rabbits will be immunized with 0.5 mg of BSA peptide (conjugated by ethyl CDI) emulsified with complete Freund's adjuvant at 2-week intervals. The antisera against peptides will then be purified by affinity chromatography coupled with CGG peptide (conjugated by morpho CDI), excluding the by-product generated in the conjugation reaction. The polyclonal antibodies will be titered (by 1:1 serial dilution, beginning with a 1:50 dilution) on human melanoma cells, either untreated or treated with IFN- $\beta$  + MEZ, by ELISA assays as described previously (91).

(ii) Immunostaining of cultured cells and sectioned patient samples with anti-*mda* peptide antibodies: Based on preliminary studies using SV40-immortalized human melanocytes, applicants would predict that specific *mda* gene products would be produced at increased levels in normal melanocytes versus melanoma cells. To test this possibility and to determine if differences are apparent in specific stages of melanoma development or as a consequence of IFN- $\beta$  + MEZ treatment, melanocytes, dysplastic nevi and different staged melanomas (RGP, early VGP, late VGP and metastatic) will be cultured on coverslips. Twenty four hr later, one group of cultures will receive a media change without additions and the other will receive fresh medium with 2000 units/ml of IFN- $\beta$  + 10 ng/ml of MEZ. After an additional 24 hr, cultures will be washed 3X with PBS followed by fixation

-171-

with 3.7% formalin for 30 min (90,91). The slides will then be washed 3X with PBS and treated with 0.2% Triton X-100/PBS for 5 min at room temperature. After extensive washing with PBS and blocking nonspecific binding sites with 2% egg albumin/PBS, the cells will be incubated with affinity purified anti-*mda* peptide antibody or preimmunized control sera for 30 min. The slides will then be incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin antibody for 30 min followed by examination with a fluorescence microscope after extensive washing with 0.1% SDS/PBS (90). Studies will also be conducted to determine if the anti-*mda* peptide antibodies react with melanocyte/melanoma lineage tissue. Reactivity of the anti-*mda* peptide antibodies toward sectioned clinical samples representing normal melanocytes, dysplastic nevi, and RGP, early VGP, late VGP and metastatic melanoma (supplied by Drs. Albino and Herlyn) (63) will be analyzed as previously described (90,91).

(iii) Analysis of the effect of forced *mda* expression on growth and differentiation in human melanoma cells: A key question will be whether expression of any of the *mda* genes can directly inhibit melanoma growth or induce morphological, biochemical or gene expression changes associated with growth suppression and the induction of differentiation without the addition of inducer (IFN- $\beta$  + MEZ). To determine the effect of overexpression of specific *mda* genes on growth and differentiation in human melanoma cells applicants will employ expression vectors containing full-length *mda* cDNAs. Full-length *mda* cDNAs will be cloned into an expression vector containing an inducible promoter, such as the dexamethasone (DEX)-inducible MMTV promoter, and a selectable antibiotic resistance gene, such as the neomycin

-172-

resistance gene, e.g., pMAMneo construct (Clonetech). Transfer of this *mda-S* construct into human melanoma cells will permit the direct isolation of cells containing the specific *mda-S* gene and will permit regulation (by altering DEX concentrations) of the level of expression of the specific *mda* gene. This approach may be necessary, since continuous increased expression of specific *mda* genes under control of promoters such as the cytomegalovirus (CMV) or the  $\beta$ -actin promoter may result in loss of proliferative ability and/or terminal differentiation in human melanoma cells. By using different MMTV promoter-driven constructs containing different *mda* genes and different antibiotic resistance genes, it will also be possible to construct melanoma cells containing several *mda-S* inducible gene constructs. An additional advantage of a regulatable expression vector system will be the stable nature of the cell clones and the ability to determine if transient or prolonged *mda-S* expression is required to induce an irreversible loss of proliferative capacity and terminal differentiation in H0-1 cells. Brief Description of Protocols: H0-1 cells (additional human melanoma cell lines or other human tumor cell lines) will be seeded at  $2 \times 10^6$  cells/100 mm plate, 24 hr later cells will be transfected by electroporation with the *mda-S* construct (alone if a selectable gene is present in the construct or in conjunction with a cloned selectable gene) as described (92). Antibiotic resistant colonies will be selected and isolated as pure clones (92, 93). The presence of the inserted gene will be determined by Southern blotting and expression of the endogenous and exogenous gene will be distinguished by RNase protection assays (58,94). The ability of DEX to enhance expression of the *mda-S* gene in appropriate H0-1 cell lines will be

-173-

determined by growing cells in the presence or absence of the appropriate inducer ( $10^{-9}$  to  $10^{-5}$  M DEX or 2000 units/ml of IFN- $\beta$ ) for 24 hr prior to isolating and characterizing RNA expression (95). Under conditions of non-induction (absence of DEX) or induction (presence of DEX) cells containing the MMTV-inducible *mda-S* constructs will be evaluated for alterations in growth (10, 14), increases in melanin synthesis (10, 69), modification in cell surface antigenic phenotype (67, 68, 72), changes in the levels of P2Ps (52, 53), patterns of gene expression (14) and the induction of irreversible loss of proliferation (terminal differentiation) (10,11,14) using previously described protocols.

Potential Outcome of *mda-S* Expression Construct Studies: The studies briefly described above could result in one of three potential outcomes. First, a single *mda-S* construct could induce growth suppression, gene expression changes associated with differentiation and terminal cell differentiation in the absence of IFN- $\beta$  + MEZ. This would provide compelling evidence that this specific *mda* gene is a controlling element in regulating growth and differentiation in human melanoma cells. Second, specific *mda-S* constructs could modify only a portion of the changes induced by IFN- $\beta$  + MEZ in human melanoma cells, i.e., induce growth suppression, induce growth suppression and some markers of differentiation or induce only some of the gene expression changes associated with differentiation without affecting growth. If this occurs, then it may be possible by using a combination of *mda-S* constructs, which induce different components of the differentiation program in human melanoma cells, to induce a loss of proliferative capacity and terminal differentiation in human melanoma

-174-

cells. Third, *mda-S* constructs could display no effects on growth or differentiation programs in H0-1 cells. This result would of course be the least informative outcome. It would suggest that the specific *mda-S* constructs tested are not controlling elements in melanoma differentiation, although these genes may be altered during the induction of growth suppression and terminal differentiation in human melanoma cells by IFN- $\beta$  + MEZ.

(iv) Analysis of the effect of antisense oligomers and expression vector constructs on growth and differentiation in human melanoma cells: Antisense RNA is an effective approach for interfering with the expression of specific target genes (96). The antisense transcript has a sequence complementary to the target mRNA and can presumably anneal to the mRNA and disrupt normal processing or translation (96). Mechanism(s) by which antisense constructs inhibit gene functions include: a direct interference in translation by binding to the ribosomal assembly (translation initiation) site and/or coding regions; stimulation of mRNA degradation by RNase H which specifically cleaves double-stranded RNA hybrids; and blocking translocation of the mRNA from the nucleus into the cytoplasm (96). Previous studies have demonstrated that antisense constructs or oligodeoxyribonucleotides (oligomers) of specific genes, such as *c-myc* and *Egr-1*, can modulate cell growth and/or differentiation (84,97-101). To determine the effect of blocking *mda* gene expression on growth and differentiation in human melanoma cells applicants will conduct experiments using antisense oligomers and expression vectors containing antisense constructs.

In the first set of experiments applicants will determine

-175-

if oligomers complementary to specific regions of the *mda* genes can induce growth suppression and/or changes in the expression of genes previously shown to be altered in H0-1 cells treated with IFN- $\beta$  + MEZ, i.e., c-myc, IL-8, FIB, MGSA/gro, ISG-15, c-jun and jun-B (14,55). As indicated above, antisense oligomers have been employed successfully to alter cell physiology in a number of cell lines and they have been shown to affect the expression of many genes (rev. 102,103). *mda*-gene specific oligomers complementary to the translation initiation sites or 5'-coding regions will be synthesized with phosphorothioate modification (phosphorothioate oligodeoxynucleotide) to increase nuclease resistance (96,102,103). Although unmodified oligomers have been utilized in many laboratories, they can be degraded rapidly by nucleases present in serum-supplemented medium (96,102). Since experiments with H0-1 and the other cell types will utilize serum-containing medium, applicants will use phosphorothioate oligodeoxynucleotides as opposed to unmodified oligomers. Brief Description of Protocols: For growth studies, H0-1 cells (or other test cell lines) will be seeded at  $2.5 \times 10^4$  cells/35mm tissue culture plate and 24 hr later fresh medium with IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml), various concentrations (1 to 200  $\mu$ M) of the 3',5'-phosphorothioate end-capped *mda* antisense oligomer complementary to several target sequences of the 5' region of the *mda* gene (20 bases in length) (*mda*-AS oligomer) or the combination of IFN- $\beta$  + MEZ and the *mda*-AS oligomer will be added. As appropriate controls, cultures will also receive similar concentrations of a 5',3'-phosphorothioate end-capped *mda* sense oligomer (*mda*-S oligomer) or the *mda*-S oligomer plus IFN- $\beta$  + MEZ (65, 84, 104). Cell numbers will be determined daily over a 7 day period (with medium changes

-176-

with the appropriate additions every 48 hr) to identify the correct *mda*-AS oligomer to use and the concentration of *mda*-AS oligomer required to block the effect of IFN- $\beta$  + MEZ on growth inhibition in H0-1 cells. If an appropriate *mda*-AS oligomer is identified it will be used for subsequent studies to determine the effect of this *mda*-AS oligomer on gene expression (14) and biochemical (10, 52, 53, 69) and immunological changes (67, 68, 72) induced in H0-1 cells treated with IFN- $\beta$  + MEZ. For gene expression studies, H0-1 cells (or other test cell lines) will be seeded at  $2.5 \times 10^6$  cells/100 mm tissue culture plate and the appropriate concentration of *mda*-AS or *mda*-S oligomer will be added 24 hr later. RNA will be isolated after an additional 24 hr or 96 hr (with a medium change after 48 hr), electrophoresed in 0.6 % agarose gels, transferred to nylon filters and hybridized sequentially with *c-myc*, *c-jun*, *jun-B*, ISG-15, MGSA/*gro*, IL-8, FIB and lastly with GAPDH (14). Assays will also be conducted to determine if *mda*-AS oligomers can prevent or diminish the biochemical, immunological and/or cellular changes induced in H0-1 cells by the combination of IFN- $\beta$  + MEZ. Parameters to be monitored, by previously described techniques, include morphology (10), melanin synthesis (10,69), antigenic expression (67,68,72) and levels of P2Ps (52,53). Possible Outcome of Studies: The experiments described above could produce one of three possible outcomes. First, specific *mda*-AS oligomers could inhibit the ability of IFN- $\beta$  + MEZ to induce growth suppression, gene expression changes associated with differentiation and terminal cell differentiation. Second, specific *mda*-AS oligomers could modify only a portion of the changes induced by IFN- $\beta$  + MEZ in H0-1 cells, i.e., reverse growth suppression, reverse both growth suppression and terminal



-177-

differentiation or reverse only some of the gene expression changes. Third, specific *mda*-AS oligomers could display no effect on the growth suppression or the differentiation program induced by IFN- $\beta$  + MEZ.

5 A positive result using *mda*-AS oligomers in blocking specific cellular and biochemical changes in H0-1 cells treated with IFN- $\beta$  + MEZ would provide strong evidence for a relationship between expression of a specific *mda* gene and a defined component of the differentiation process. However, a negative effect of a specific *mda*-AS gene could occur for many reasons including lack of stability of the antisense oligomer, inadequate quantity of the antisense oligomer or the requirement for the expression of multiple *mda* genes in the differentiation process. To further explore the effect of *mda*-AS gene expression in inhibiting chemical induction of differentiation in human melanoma cells experiments will also be conducted using *mda*-AS cDNAs (*mda* cDNAs cloned in an antisense orientation) in expression vector constructs. Since one question applicants intend to address is the relationship between levels of expression and tissue specific expression of specific *mda*-AS cDNAs and cellular phenotype, applicants will use several expression vector constructs under the transcriptional control of different promoters. The constructs to be used should result in high level targeted, constitutive or inducible transcriptional control of the *mda*-AS cDNAs. The same *mda*-AS cDNA will be cloned into an expression vector containing a promoter which will permit: high levels of constitutive expression ( $\beta$ -actin promoter (84,94)); enhanced expression after interferon treatment (interferon responsive sequence (IRS) promoter (pTKO-1) (105); inducible expression in the presence of dexamethasone- (MMTV) inducible promoter (93, 106); or

-178-

expression in melanocyte/melanoma lineage cells (tyrosinase promoter (107)). In addition, by using different selectable genes, i.e., neomycin, histidinol, hygromycin etc., either present in the expression vector construct or by cotransfection (93) it will be possible to construct human melanoma cells which contain several *mda*-AS cDNAs. In summary, the use of different promoters will permit a direct evaluation of constitutive, inducible and cell-lineage specific (targeted) expression of the *mda*-AS cDNA on growth and differentiation in human melanoma and other cell types. If expression of a specific *mda*-AS cDNA inhibits the ability of IFN- $\beta$  + MEZ to induce growth suppression, changes in gene expression and terminal differentiation in H0-1 and other human melanoma cells, this would provide strong evidence for a direct relationship between expression of this *mda* cDNA and the growth and differentiation process in human melanoma cells. Brief Description of Protocols: H0-1 cells (additional human melanoma cell lines or other human tumor cell lines) will be seeded at  $2 \times 10^6$  cells/100 mm plate, 24 hours later cells will be transfected by electroporation with the *mda*-AS construct (alone if a selectable gene is present in the construct or in conjunction with a cloned selectable gene) as described (92, 93). Antibiotic resistant colonies will be selected and isolated as pure clones (92, 93). The presence of the inserted gene will be determined by Southern blotting and expression of the endogenous and exogenous gene will be distinguished by RNase protection assays (58, 94). The ability of interferon or dexamethasone to enhance antisense in a pTKO-1 (interferon inducible promoter) or a pMAMneo (DEX inducible) construct, respectively, will be determined by growing cells in the presence or absence of the

-179-

appropriate inducer ( $10^{-9}$  to  $10^{-5}$  M DEX or 2000 units/ml of IFN- $\beta$ ) for 24 hours prior to isolating and characterizing RNA expression (95, 105). Similar cellular (growth and morphology), biochemical (melanin and P2P levels), immunological (antigenic expression) and molecular (gene expression) parameters as used to study *mda-S* constructs will be used to study *mda-AS* constructs.

Summary: These studies will provide important information about the *mda* encoded gene products and they will indicate if perturbations in the expression of specific *mda* genes can directly modify growth or the differentiation process in human melanoma cells.

D. Specific Aim #4: Isolate and characterize the promoter region of *mda* genes and analyze their regulation in human melanocytes, nevi and melanoma.

1. Rationale and General Approach:

In order to elucidate the mechanism underlying the transcriptional regulation of the *mda* genes it will be necessary to analyze the promoter regions of these genes. This will be important for studies aimed at determining regulatory control of the *mda* genes including chemical induction, autoregulation, tissue specific regulation and developmental regulation. Once the appropriate promoters of the *mda* genes have been isolated, studies can be conducted to identify relevant trans-regulatory factors (nuclear proteins) which bind to specific cis-regulatory elements and activate or repress the expression of the *mda* genes. The experiments outlined below are designed to: [a] clone the promoter region of specific *mda* genes and analyze their function in untreated and IFN- $\beta$  + MEZ

-180-

treated melanoma cells; [b] identify cis-regulatory elements in the promoter region of specific *mda* genes which are responsible for IFN- $\beta$  + MEZ induction in human melanoma cells; and [c] identify and characterize trans-regulatory elements which activate (or repress) expression of the *mda* genes.

(a) Cloning the promoter region of the *mda* genes and testing their function in untreated and IFN- $\beta$  + MEZ treated human melanoma cells: To isolate the promoter region of the *mda* gene a human genomic library will be constructed by partial digestion of H0-1 human melanoma genomic DNA with the restriction enzyme Sau3AI and then ligation into dephosphorylated vectors (phage or cosmid vectors) (92). Using the *mda* cDNA gene to screen the library, clones will be identified which contain both the *mda* gene and its 5' and 3' regions (92). Since the insert in a phage or cosmid vector is too large to analyze (i.e., 10 to 30 Kb) and the structure and size of the genomic DNA for the *mda* genes are not known, the inserts will be subcloned (to an approximate size of 2 Kb) in order to identify the potential promoter region (92). Two types of probes will be used for subcloning: one containing the coding region of the *mda* gene and the other a synthetic oligonucleotide (a 20 mer) complementary to the sequence located in the 5' non-translated region of the *mda* gene. The genomic DNA containing the *mda* gene in phage or cosmid vector will be digested with a series of restriction enzymes, electrophoresed on 0.8% agarose gels and transferred to nylon filters (92,108). This Southern blot will then be probed with the coding region of the *mda* gene and the synthetic oligonucleotide. This double probing method will permit a more effective identification of the promoter region than utilizing a single probe. Putative

-181-

promoter inserts of approximately 2 Kb in size will be subcloned into various CAT expression vector constructs (including pSVOCAT, pUVOCAT or pChlorAce) for later functional analysis (92,108,109).

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The putative promoter region of the *mda* genes will be sequenced by the Sanger dideoxynucleotide procedure (110). The transcription start site (+1) will be determined by primer extension as described previously (108, 111). Dried total RNA samples of H0-1 melanoma cells with or without treatment with IFN- $\beta$  + MEZ will be resuspended in 20  $\mu$ l of 10 mM PIPES (pH 6.4)-400 mM NaCl containing 5'-end  $^{32}$ P-labeled oligonucleotides (a 25 mer, complementary to the 5'-untranslated region of the *mda* gene) made by the T4 nucleotide kinase method. After 3 hr incubation at 60°C, 80  $\mu$ l of 50 mM Tris-HCl (pH8.2)-5 mM MgCl<sub>2</sub>-10 mM dithiothreitol-5 mM deoxyguanosine nucleoside triphosphates-25  $\mu$ l of dactinomycin per ml containing 10 U of avian myeloblastosis virus reverse transcriptase will be added and the primer extension reaction will be allowed to proceed for 1 hr at 42°C. Following phenol-chloroform extraction and ethanol precipitation, samples will be electrophoresed on 6% acrylamide-8 M urea sequencing gels (108,111). From the length of the extended products, the transcription initiation site of the *mda* gene can be determined.

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To functionally analyze the various *mda* promoters, appropriate *pmdaCAT* constructs will be transfected into melanoma cells by the CaPO<sub>4</sub> method or electroporation (Gene Pulser, Bio-Rad) as previously described (93, 108, 109). Cell extracts will be prepared 48 hr after transfection by washing cells 3X with PBS, pelleting manually resuspended cells and lysing cells by three

-182-

cycles of freeze-thawing. The CAT reaction will then be performed by adding 55  $\mu$ l of cell extracts into a reaction mixture consisting of 5  $\mu$ l of  $^{14}$ C-chloramphenicol, 70  $\mu$ l of 1 M Tris-HCl (pH8.0) and 20  $\mu$ l of 4 mM butyryl CoA (108,109). After incubation at 37°C for 2 hr, the reaction mixture will be extracted with ethyl acetate or xylene and CAT activity will be determined either by scintillation counting or by TLC (108,109). If CAT expression can be detected in specific *pmda*CAT construct transfected human melanoma cells after treatment with IFN- $\beta$  + MEZ, but not in untreated cultures, this would indicate that the promoter region of the specific *mda* gene contains appropriate cis-acting elements responsive to induction by IFN- $\beta$  + MEZ. If no induction is apparent, further subcloning and screening of cosmid or phage clones would be performed until an *mda* promoter of sufficient length to mediate CAT induction in differentiation inducer-treated human melanoma cells is obtained. A potentially useful series of CAT constructs have been developed by United States Biochemical (Cleveland, Ohio), referred to as the pChlorAce series. The basic plasmid pChlorAce-B does not contain a eucaryotic promoter or enhancer sequences and is therefore dependent on incorporation of a functional promoter upstream from the CAT gene for expression of CAT activity. The construct, pChlorAce-E, contains an enhancer sequence permitting a direct test of a functional promoter CAT-junction for testing *mda* promoter sequences. The promoter containing plasmid, pChlorAce-P, incorporates an SV40 promoter upstream from the CAT gene allowing the insertion of enhancer elements in both orientations upstream or downstream from the promoter-CAT transcriptional unit. It will be possible by inserting different parts of the *mda* gene into the pChlorAce-P

-183-

construct to directly identify the enhancer component of the *mda* gene. The control plasmid, pChlorAce-C contains both the promoter and enhancer and can function as an internal standard for comparing promoter and enhancer strengths.

An important question will be whether specific *mda* genes display tissue- and developmental-specific expression. Once specific *mda* promoters are identified they can be used to address this issue. *mda*-CAT constructs will be tested for levels of expression in untreated and IFN- $\beta$  + MEZ-treated normal human melanocytes, dysplastic nevi and RGP, early VGP, late VGP and metastatic melanomas. Experiments will also be performed to determine if the *mda* promoters can function in either untreated or IFN- $\beta$  + MEZ-treated non-melanocyte/melanoma lineage cells. Positive expression in specific target cells would suggest that the appropriate regulatory proteins are either constitutively present or inducible in these cells.

(b) Identifying cis-elements in the *mda* promoter responsible for induction by IFN- $\beta$  + MEZ in human melanoma cells: Once a functional *mda* promoter has been identified studies will be conducted to locate cis-elements responsible for induction of expression by IFN- $\beta$  + MEZ. The approach to be used will involve the construction and evaluation for CAT inducible activity of a series of 5'-deletion mutants, 3'-deletion mutants, internal-deletion mutants and linker-scanning mutants of the *mda* promoter regions. The details for constructing the 5'-deletion, 3'-deletion and internal-deletion mutants and screening for CAT activity has been described previously (108,109). Linker-scanning mutants will be constructed by combining 5'- and 3'-deleted *mda* promoter

-184-

sequences after filling gap regions with a linker DNA sequence (such as an EcoRI linker) (92). The structure of the various mutants will be determined by sequence analysis (95,110). Since the promoter region for the *mda* gene is located in front of the CAT reporter gene in the various *pmdaCAT* constructs, the CAT activity for each construct can be measured using liquid scintillation and/or TLC (108, 109). This will permit a direct comparison of CAT transcriptional activity of the mutant promoter to that of the unmodified *mda* promoter. These studies will result in the identification of specific cis-regulatory elements responsible for IFN- $\beta$  + MEZ induction of enhanced *mda* gene expression in human melanoma cells.

(c) Identifying trans-acting nuclear proteins induced by IFN- $\beta$  + MEZ which mediate transcriptional enhancing activity of *mda* genes in human melanoma cells: The current view on regulation of eucaryotic gene expression suggests that trans-acting proteins bind to specific sites within cis-elements of a promoter region resulting in transcriptional activation (rev in 112,113). Studies employing various mutant *mda* promoter CAT constructs will provide information relative to cis-regulatory elements mediating activity of the *mda* promoters. Experiments will be performed to identify trans-acting factors (nuclear proteins) and determine where these factors interact with cis-regulatory elements. To achieve this goal, two types of studies will be performed, one involving DNase-I footprinting (methylation interference) assays (108,109) and the second involving gel retardation (gel shift) assays (109,114).

For DNase-I footprinting assays nuclear extracts from human melanoma cells, untreated or treated with IFN- $\beta$  +



-185-

MEZ, will be prepared as described previously (109,113,115). DNase-I footprinting assays will be performed as described (108,109). The cis-element (approximately 200 bp) for IFN- $\beta$  + MEZ induction, identified from the experiments described above, will be terminally labeled with  $^{32}\text{P}$  and incubated with crude nuclear extracts from untreated or IFN- $\beta$  + MEZ treated human melanoma cells using the protocols described previously (108,109). The reaction mixture which has been digested with DNase-I enzyme will be terminated and the digested products will be analyzed on an 8% sequencing gel (108,109). The differential protection between nuclear extracts from induced and uninduced human melanoma cells will provide relevant information concerning the involvement of trans-acting factors in activation and the location of specific sequences in the cis-regulatory elements of the *mda* promoter mediating this activation. If differential protection is not detected using this approach, the sensitivity of the procedure can be improved by using different sized DNA fragments from the *mda* promoter region or by using partially purified nuclear extracts (109).

As a second approach for investigating the interaction between cis-acting elements in the *mda* promoter and trans-acting factors in mediating transcriptional control, gel shift assays will be performed as described previously (109,114). For this assay,  $^{32}\text{P}$ -labeled cis-elements will be incubated with nuclear extracts from untreated or IFN- $\beta$  + MEZ treated human melanoma cells and reaction mixtures will be resolved on 5 or 8% polyacrylamide gels (109,114). After autoradiography, the pattern of retarded DNAs on the gel will provide information concerning the interaction between

-186-

trans-acting factors and specific regions of the cis-elements in the *mda* promoters. Non-labeled cis-elements (self-competition) will be added as a competitor to duplicate samples to eliminate the possibility of non-specific binding and to confirm that the interaction is really conferred by the trans-acting factor. To begin to identify the trans-acting factors, different non-labeled DNAs (for example TATA, CAT, TRE, Sp-I binding site, NFkB, CREB, TRE, TBP, etc.) can be used as competitors in the gel shift assay to determine the relationship between the trans-acting factors and other previously identified transcriptional regulators.

Summary: These studies will result in the identification and cloning of the *mda* promoter region, the identification of cis-regulatory elements in the *mda* promoters and the identification of trans-regulatory elements which activate (or repress) expression of *mda* genes.

F. Future Studies: The currently proposed research will result in the characterization of specific genes which may be involved in or mediate growth control, response to chemotherapeutic and DNA damaging agents and terminal differentiation in human melanoma cells. Once appropriate *mda* genes are identified they can subsequently be used to directly test their functional role in development and melanocyte/melanoma biology. Experiments can also be performed to define the role of these genes in non-melanoma target cells and additional programs of differentiation. Future studies which also are not within the scope of the present proposal would include: (a) evaluation of the effect of specific *mda-S* and *mda-AS* constructs on the growth (tumorigenic and metastatic potential) and differentiation of human

-187-

melanoma (and other tumor cell types) grown in vivo in nude mice; (b) generation of transgenic mice displaying both tissue and non-tissue specific overexpression of individual or combinations of *mda* genes to evaluate the effect of modified *mda* expression on development; and (c) homologous recombination-mediated gene targeting techniques to produce mice with specific *mda* null mutations to evaluate the effect of lack of expression of specific *mda* genes on tissue and embryo development.

-188-

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-201-

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-207-

Fourth Series of Experiments

mda-1: Novel gene which displays increased expression in  
IFN- $\beta$  and IFN- $\beta$  + MEZ treated H0-1 cells after 24 hours.  
5 Decreased expression occurs in H0-1 cells treated with  
IFN- $\beta$  + MEZ for 96 hours. (HJ 3-13).

TGGA CT TGT GTT CTG ACT AGA ACT CAAC ATG TTACT AGG CAC ATG TGT CAT GTCT  
CAGG TCAG TGCT GTG ACAGA ATTG ATACG AGAG AAATG TCG CT TATG CTATCA CT  
10 GATCTACACATGTCTGATAGATAGTCAGATACAGATGATGAGGAATCT (Seq. ID  
No. 1)

mda-2:

15 GAATTCAGTGA ACTCTTTTCTCATTCTCTTTGTTTTGTGGCACTTCACAATGTAG  
AGGAAAAAACCAATGACCGCACTGTGATGTGAATGGCACC GAAGTCAGATGAGT  
ATCCTGTAGGTCACCTGCAGCCTGGCTTGCCACTTGTCTTA ACTCTGAATATTTT  
ATTTCAAAGGTGCTAAAATCTGAAATCTGCTAGTGTGAACTTGCTCTACTCTCTG  
AATGATTCAATCCTATT CATACTATCTTGTAGATATATCAACTAAAAAAA (Seq.  
20 ID No. 2)

Properties of mda-4

This cDNA is novel (analysis of various gene data bases  
25 indicates that the sequence of mda-4 is 68.5% homologous  
to the human interferon gamma induced protein).

Expression in H0-1 Human Melanoma Cells

30 Increased expression after 24 hour treatment of H0-1  
cells with recombinant human fibroblast interferon (IFN- $\beta$ )  
(2,000 units/ml), mezerein (MEZ) (10 ng/ml) and to a  
greater extend with the combination of IFN- $\beta$  + MEZ (2,000  
units/ml + 10 ng/ml).

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-208-

Analysis of terminally differentiated H0-1 cells, i.e., H0-1 cells treated with the combination of IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml) for 96 hours, indicate continued increased expression in IFN- $\beta$  + MEZ treated H0-1 cells.

Increased expression in H0-1 cells after 96 hour exposure to immune interferon (IFN- $\gamma$ ) (2,000 units/ml) and IFN- $\beta$  + IFN- $\gamma$  (1,000 units/ml + 1,000 units/ml) (note: this combination of agents results in a similar degree of growth suppression in H0-1 cells as does IFN- $\beta$  + MEZ. However, growth suppression is reversible with the combination of interferons, whereas it is irreversible with the combination of IFN- $\beta$  + MEZ).

*mda-4* represents a novel IFN- $\gamma$ -inducible gene which is also induced during terminal cell differentiation in H0-1 cells. Could prove useful as a gene marker for immune interferon response and as a gene marker for terminal differentiation in human melanoma cells.

Expression in Additional Human Melanoma Cells

Increased expression of *mda-4* results after a 24 hour treatment with IFN- $\beta$  + MEZ in H0-1, L0-1, SH-1, WM278 and WM239 human melanoma cells. *Mda-4* is not expressed or inducible in the melanotic F0-1 human melanoma cell or in the C8161 human melanoma cells or C8161/6.3 cells (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells, they are non-metastatic).

*Mda-4* displays increased expression in additional human melanoma cells besides the human melanoma cell from which it was cloned, i.e., H0-1 after 24 hour treatment with



-209-

IFN- $\beta$  + MEZ.

5     Expression in Normal Cerebellum, a Central Nervous System  
Tumor (Glioblastoma Multiforme) (GBM) and Normal Skin  
Fibroblast Cell Lines

10     *Mda-4* is not expressed de novo in normal cerebellum, GBM  
or normal skin fibroblasts. However, it is inducible in  
both normal cerebellum and GBM, but not in normal skin  
fibroblasts, following a 24 hour treatment with IFN- $\beta$  +  
MEZ.

15     *Mda-4* is susceptible to induction by IFN- $\beta$  + MEZ in human  
cerebellum and GBM cultures, but not in normal human skin  
fibroblasts.

20     Expression in Colorectal Carcinoma (SW613), Endometrial  
Adenocarcinoma (HTB113) and Prostate Carcinoma (LNCaP)

20     *Mda-4* is not expressed de novo in various carcinoma cells  
and it is not inducible in these cells following a 24  
hour treatment with IFN- $\beta$  + MEZ. *Mda-4* is not expressed  
in human carcinoma cells.

25     Effect of Various Treatment Protocols on Expression in  
H0-1 Cells

30     A 24 hour treatment with IFN- $\beta$  (2,000 units/ml), IFN- $\alpha$   
(2,000 units/ml), IFN- $\beta$  + MEZ (2,000 units/ml + 10  
ng/ml), IFN- $\alpha$  + MEZ (2,000 units/ml + 10 ng/ml), cis-  
platinum (0.1  $\mu$ g/ml), gamma irradiation (treated with 3  
gray and analyzed after 24 hours). In addition,  
treatment with UV (10 joules/mm<sup>2</sup> and assayed 24 hours  
later) results in increased expression in H0-1 cells.

-210-

No or only a small change in expression is observed in H0-1 cells treated with MEZ (10 ng/ml; 24 hours or 96 hours), IFN- $\beta$  (2,000 units/ml; 96 hours), phenyl butyrate (PB) (4 mM; treated for 24 hours, 4 days or 7 days), mycophenolic acid (MPA) (3  $\mu$ M; 96 hours), trans retinoic acid (RA) (2.5  $\mu$ M; 24 hours), MPA + MEZ (3  $\mu$ M + 10 ng/ml; 96 hours), RA + MEZ (2.5  $\mu$ M; 96 hours), actinomycin D (5  $\mu$ g/ml for 2 hours, assayed after 24 hours), adriamycin (0.1  $\mu$ g/ml; 24 hours), vincristine (0.1  $\mu$ g/ml; 24 hours), TNF- $\alpha$  (100 units/ml; 24 hours) or VP-16 (5  $\mu$ g/ml; 24 hours).

*mda-4* is a novel gene which displays the following properties: 1) it is inducible in H0-1 cells during terminal differentiation (treatment with IFN- $\beta$  + MEZ for 96 hours) and following 96 hour treatment with recombinant gamma interferon (alone or in combination with IFN- $\beta$ ); 2) it is inducible by IFN- $\beta$  + MEZ in a series of human melanomas in addition to H0-1, normal cerebellum and GBM cells, but it is not expressed or inducible by IFN- $\beta$  + MEZ in normal skin fibroblasts or three different types of carcinomas (colorectal, endometrial adenocarcinoma or prostate carcinoma); and 3) increased expression is induced in H0-1 cells treated with specific DNA damaging agents (cis-platinum, gamma irradiation and UV irradiation).

This gene represents a cytokine-, DNA damage-, and chemotherapy- (cis-platinum) and terminal differentiation-inducible gene possibly restricted to cells of neuroectodermal origin (melanoma and central nervous system). *mda-4* may prove useful: 1) as a marker for specific tissue lineage's (i.e., neuroectodermal) (diagnostic applications); 2) to monitor response to DNA damage (induced by gamma irradiation and UV irradiation)

-211-

and treatment with chemotherapeutic agents which work in a similar manner as cis-platinum (diagnostic applications); 3) to assay for types I (IFN- $\alpha$  and IFN- $\beta$ ) and type II (IFN- $\gamma$ ) interferon in biological fluids (diagnostic applications); and 4) to identify compounds which have the capacity to induce terminal differentiation in human melanoma cells (drug screening programs to identify new chemotherapeutic agents). Once full-length cDNAs are isolated, this gene (used in a sense orientation in an appropriate expression vector) may prove useful in inhibiting growth and inducing terminal differentiation in human melanomas and specific central nervous system tumors (GBM) (therapeutic applications). Antisense constructs of specific regions of this gene could also prove useful in preventing damage to normal tissue (e.g., bone marrow) treated with differentiation inducing and specific chemotherapeutic and DNA damaging agents (therapeutic applications).

#### 20 mda-4

TTCTTCTTTGTAAAAGTTTTTAATACACTGCTGAAAGATAAATTCATTCCAAAGA  
GAATAATTATATAGCAAGATATTATCGGCACAGTGGTTTCTTAGAGGTAAATAGCG  
CCTCACGTGTGTTAGATGCTGAATCTGACCAA (SEQ ID No. 5)

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#### Properties of mda-5

This cDNA is novel (it has sequence homology to a Homo sapiens putatively transcribed partial sequence; Accession number Z20545; from the UK-HGMP, MRC Human Genome Mapping Project Resource, Centre Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 England)

#### Expression in H0-1 Human Melanoma Cells

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-212-

Increased expression after 24 hour treatment of H0-1 cells with recombinant human fibroblast interferon (IFN- $\beta$ ) (2,000 units/ml), and to a greater extent with IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml) in combination.

5

Analysis of terminally differentiated H0-1 cells, i.e., H0-1 cells treated with the combination of IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml) for 96 hours, indicate continued increased expression in IFN- $\beta$  + MEZ treated H0-1 cells.

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Enhanced expression of *mda-5* is also observed, albeit to a lesser extent, in H0-1 cells treated for 96 hours with immune interferon (IFN- $\gamma$ ) (2,000 units/ml) and in 96 hours IFN- $\beta$  + IFN- $\gamma$  (1,000 units/ml + 1,000 units/ml) treated H0-1 cells (greater increased than with IFN- $\gamma$  alone) (note: this combination of agents results in a similar degree of growth suppression in H0-1 cells as does IFN- $\beta$  + MEZ. However, growth suppression is reversible with this combination of interferons, whereas it is irreversible with the combination of IFN- $\beta$  + MEZ).

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*mda-5* represents a novel IFN- $\gamma$ -inducible gene which also displays increased expression during terminal cell differentiation in H0-1 cells. This gene could prove useful as a marker for immune interferon response and as a marker for terminal differentiation in human melanoma cells.

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#### Expression in Additional Human Melanoma Cells

Increased expression of this gene occurs in H0-1, C8161, C8161/6.3 (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells

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-213-

they are non-metastatic), F0-1, L0-1, SH-1, WM278 and WM239 human melanoma cells treated with IFN- $\beta$  + MEZ for 24 hours. This gene is constitutively expressed in immortalized human melanocytes FM5169 (transformed by SV40). Some upregulation is observed in FM5169 following IFN- $\beta$  + MEZ treatment for 24 hours.

Expression of *mda-5* is increased by IFN- $\beta$  + MEZ in 7 additional human melanoma cells besides the human melanoma cell it was cloned from i.e., H0-1. In addition, this gene is expressed in melanocytes and its expression is increased to a lesser degree than in most human melanomas following a 24 hour treatment with IFN- $\beta$  + MEZ.

15

Expression in Normal Cerebellum, a Central Nervous System Tumor (Glioblastoma Multiforme) (GBM) and Normal Skin Fibroblast Cell Lines

*mda-5* is express de novo at low levels in normal cerebellum, but not in GBM or normal skin fibroblasts. However, expression is increased in normal cerebellum (>10-fold) and expression is induced in GBM (small induction) and normal skin fibroblasts (good induction) following a 24 hour treatment with IFN- $\beta$  + MEZ.

This gene is susceptible to modulation by IFN- $\beta$  + MEZ in human cerebellum, GBM and normal human skin fibroblasts. Differential de novo and inducible expression is seen in normal cerebellum cells versus GBM, with normal cerebellum displaying both higher de novo and inducible expression.

Expression in Colorectal Carcinoma (SW613), Endometrial Adenocarcinoma (HTB113) and Prostate Carcinoma (LNCaP)

-214-

*mda-5* is not expressed de novo in colorectal carcinoma (SW613) and endometrial adenocarcinoma (HTB113), whereas it is expressed at low levels in prostate carcinoma (LNCaP).

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Following a 24 hour treatment with IFN- $\beta$  + MEZ, *mda-5* expression is induced at high levels in colorectal carcinoma (SW613) cells, but no expression is seen in endometrial adenocarcinoma (HTB113).

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In the case of human prostate (LNCaP), IFN- $\beta$  + MEZ treatment for 24 hours results in a 2- to 3-fold increase in mRNA expression.

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This gene displays a differential pattern of both de novo and inducible expression in different human carcinomas.

Effect of Various Treatment Protocols on Expression in H0-1 Cells

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A 24 hour treatment with IFN- $\beta$  (2,000 units/ml), IFN- $\alpha$  (2,000 units/ml), IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml) and IFN- $\alpha$  + MEZ (2,000 units/ml + 10 ng/ml) results in increased expression in H0-1 cells.

25

*mda-5* is induced after 96 hour treatment with IFN- $\gamma$  and IFN- $\beta$  + IFN- $\gamma$ . Highest level of expression is observed in H0-1 cells treated with IFN- $\beta$  + MEZ for 24 or 96 hours. Very low induction after 96 hour treatment with

30

IFN- $\beta$  (2,000 units/ml).

No change in expression is observed in H0-1 cells treated with MEZ (10 ng/ml) (24 or 96 hours), MPA (3  $\mu$ M; 96 hours), RA (2.5  $\mu$ M; 96 hours), MPA + MEZ (3  $\mu$ M + 10

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ng/ml; 96 hours), RA + MEZ (2.5  $\mu$ M + 10 ng/ml; 96 hours),

-215-

phenyl butyrate (PB) (4 mM PB for 24 hours, 4 days or 7 days), cis-platinum (0.1  $\mu$ g/ml; 24 hours), gamma irradiation (treated with 3 gray and analyzed after 24 hours), UV (10 joules/mm<sup>2</sup>, assayed 24 hours later),  
5 actinomycin D (5  $\mu$ g/ml for 2 hours, assayed 24 hours later), adriamycin (0.1  $\mu$ g/ml; 24 hours), vincristine (0.1  $\mu$ g/ml; 24 hours), cis-platinum (0.1  $\mu$ g/ml; 24 hours), TNF- $\alpha$  (100 units/ml; 24 hours) or VP-16 (5  $\mu$ g/ml; 24 hours).

10

*mda-5* is a novel gene which displays the following properties: 1) it is inducible during terminal differentiation (treatment with IFN- $\beta$  + MEZ for 96 hours) and following treatment for 96 hours with recombinant  
15 gamma interferon (alone or in combination with IFN- $\beta$ ); 2) treatment for 24 hours with IFN- $\beta$  + MEZ results in increased expression in all human melanomas tested and in an SV40-immortalized human melanocyte; 3) it is highly inducible by IFN- $\beta$  + MEZ within 24 hours in normal  
20 cerebellum and normal skin fibroblast cells, but it is only weakly inducible in GBM; 4) it is differentially inducible in three different types of carcinomas (with induction greatest in colorectal, low induction in prostate carcinoma and no induction in endometrial  
25 adenocarcinoma); and 5) increased expression is induced in H0-1 cells treated with both type I interferon (IFN- $\alpha$  and IFN- $\beta$ ) and type II interferon (IFN- $\gamma$ ) (IFN- $\beta$  is more effective than IFN- $\alpha$  when used at an equivalent dose in enhancing expression of this gene).

30

This gene represents a cytokine- and terminal differentiation-inducible gene displaying increased expression in all melanomas, in select carcinomas, in normal skin fibroblasts and in both normal cerebellum and

-216-

GBM. *mda-5* may be useful: 1) as a marker for specific tissue lineages and for distinguishing tumors of similar histotype (i.e., carcinomas) (diagnostic applications; 2) to monitor response to type I and II interferon treatment (diagnostic applications; and 3) to identify compounds which have the capacity to induce terminal differentiation in human melanoma cells (drug screening programs to identify new differentiation-inducing agents). Once full-length cDNAs are isolated, this gene (used in a sense orientation in an appropriate expression vector) may prove useful in inhibiting growth and inducing terminal differentiation in human melanomas and other classes of tumors (therapeutic applications).

15 *mda-5*

CTGCAAAGAAGTGTGCCGACTATAAATAAATGGTGAAATCATCTGCAAATGTGG  
CCAGGCTTGGGGAACAATGATGGTGACAAAGGCTTAGATTTGCCTTGTCTCAAA  
ATAAGGAATTTGTAGTGGTTTCAAATATCACAAGAACGTACAAGTGGTAGATA  
20 CTATCACATTCAGTACTATCAGAGTCG (SEQ ID No. 6)

ACAAACCAGTGATTCCCCTTCCTCAGATACTGGGACTAACAGCTTCACCTGGTGT  
TGGAGGGGCCACGAAGCAAGCCAAAGCTGAAGAACACATTTTAAACTATGTGCC  
TATCTTGATGCATTTACTATTAAACTGTTAAAGAAAACCTTGATCAACTGAAAA  
25 ACCAAATACAGGAGCATGCAAGAAGTTTGCCATTGCAGATGCAACCAGAGAAGAT  
CCATTTAAAGAGAACTTCTAGAAATAATGACAAGGATTCAAACCTTATTGTCAAA  
TGAGTCCAATGTCAGATTTTGGACTC (Seq. ID No. 7)

30 Properties of *mda-6*

*mda-6* is identical to WAF1, CIP1, SDI1 that encodes a Mr.21,000 protein (p21) that is an inhibitor of cyclin dependent kinases.

35 Expression on H0-1 Human Melanoma Cells



-217-

Increased expression after 24 hour treatment of H0-1 cells with recombinant human fibroblast interferon (IFN- $\beta$ ) (2,000 units/ml), MEZ (10 ng/ml) and to the greatest extent with IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml) in combination.

Analysis of terminally differentiated H0-1 cells, i.e., H0-1 cells treated with the combination of IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml) for 96 hours indicate continued increased expression in IFN- $\beta$  + MEZ treated H0-1 cells; and (b) no significant or reduced alteration in expression after 96 hour treatment in H0-1 cells treated with IFN- $\beta$  (2,000 units/ml), IFN- $\gamma$  (2,000 units/ml), MEZ (10 ng/ml), mycophenolic acid (MPA) (3  $\mu$ M) (induces growth suppression increased melanin synthesis and morphology changes, but not terminal differentiation in H0-1 cells) trans retinoic acid (RA) (2.5  $\mu$ M) (increases melanin synthesis and tyrosinase activity, but does not alter growth, morphology or induce terminal differentiation in human melanoma cells), IFN- $\beta$  + IFN- $\gamma$  (1,000 units/ml + 1,000 units/ml) (growth suppressive without inducing markers of differentiation), MPA + MEZ (3  $\mu$ M + 10 ng/ml) (reversible growth suppression and induction of differentiation markers in H0-1 cells) and RA + MEZ (2.5  $\mu$ M + 10 ng/ml) (growth suppression and reversible induction of differentiation markers without inducing terminal cell differentiation).

*mda-6* represents a novel gene which is enhanced in H0-1 cells by IFN- $\beta$  + MEZ (which induces terminal differentiation) but not to the same extent by agents inducing growth suppression or various markers of differentiation. It could prove useful as a marker for the induction of terminal differentiation in human melanoma cells.

-218-

Expression in Additional Human Melanoma Cells

Variable increases in expression of this gene occur in H0-1, C8161, C8161/6.3 (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells they are non-metastatic), F0-1, L0-1, SH-1, WM278 and WM239 human melanoma cells treated with IFN- $\beta$  + MEZ for 24 hours. This gene is constitutively expressed in immortalized human melanocytes FM516-SV (transformed by SV40). Some upregulation is also observed in FM516-SV following IFN- $\beta$  + MEZ treatment for 24 hours.

Expression of *mda-6* is increased by IFN- $\beta$  + MEZ in 7 additional human melanoma cells besides the human melanoma cell it was cloned from, i.e., H0-1. In addition, this gene is expressed in melanocytes and its expression is increased following a 24 hour treatment with IFN- $\beta$  + MEZ.

Expression in Normal Cerebellum, a Central Nervous System Tumor (Glioblastoma Multiforme) (GBM) and Normal Skin Fibroblast Cell Lines

*mda-6* is expressed de novo at high levels in normal cerebellum and normal skin fibroblasts. *mda-6* is not expressed at significant levels in GBM cells. *mda-6* expression is increased in normal cerebellum (>10-fold) and expression is induced in GBM following a 24 hour treatment with IFN- $\beta$  + MEZ. *mda-6* expression is not altered in normal human skin fibroblasts after a 24 hour treatment with IFN- $\beta$  + MEZ.

This gene is susceptible to modulation by IFN- $\beta$  + MEZ in

-219-

human cerebellum and GBM. In contrast, this gene is expressed and no change in expression is seen following treatment in normal human skin fibroblasts. Differential de novo and inducible expression is also apparent in  
5 normal cerebellum cells versus GBM, with normal cerebellum displaying higher de novo expression. This gene could be a component of growth control in central nervous system glial cells (including normal cerebellum cells), which is repressed in malignant GBM cells.

10 Expression in Colorectal Carcinoma (SW613), Endometrial Adenocarcinoma (HTB112) and Prostate Carcinoma (LNCaP)

*mda-6* is expressed at high levels de novo in colorectal  
15 carcinoma (SW613) and prostate carcinoma (LNCaP). *mda-6* de novo expression in endometrial adenocarcinoma (HTB113) is low. Treatment for 24 hours with IFN- $\beta$  + MEZ does not significantly alter *mda-6* expression in colorectal carcinoma (SW613) or prostate carcinoma (LNCaP).  
20 Treatment for 24 hours with IFN- $\beta$  + MEZ induces *mda-6* expression in endometrial adenocarcinoma (HTB113) to a similar level as in colorectal and prostate carcinomas.

*mda-6* displays a differential pattern of both de novo and  
25 inducible expression in different human carcinomas. De novo expression is low in endometrial adenocarcinoma and high in colorectal and prostate carcinoma. Inducible expression following treatment with IFN- $\beta$  + MEZ is only observed in endometrial adenocarcinoma cells.

30 Effect of Various Treatment Protocols on Expression in H0-1 Cells

Treatment with IFN- $\beta$  (2,000 units/ml; 24 hours), MEZ (10  
35 ng/ml; 24 hours), IFN- $\beta$  + MEZ (2,000 units/ml + 10 ng/ml;

-220-

24 hours), actinomycin D (5  $\mu\text{g/ml}$ ; 2 hour treatment followed by 24 hour growth), adriamycin (0.1  $\mu\text{g/ml}$ ; 24 hour) and VP-16 (5  $\mu\text{g/ml}$ ; 24 hour) results in increased expression in H0-1 cells. Highest level of induction observed in H0-1 cells treated with IFN- $\beta$  + MEZ for 24 hours or 96 hours; and actinomycin D, adriamycin and VP-16 treated for 24 hours.

Decreased expression of *mda-6* is observed in H0-1 cells treated with phenyl butyrate (PB) (4 mM) (24 hours, 4 or 7 days), cis-platinum (0.1  $\mu\text{g/ml}$ ; 24 hours), UV (10 joules/ $\text{mm}^2$ ; 2 hours after treatment), gamma irradiation (3 gray; assayed after 24 hours), IFN- $\alpha$  (2,000 units/ml; 24 hours) and TNF- $\alpha$  (100 units/ml; 24 hours).

No change in *mda-6* expression observed in H0-1 cells treated with UV (10 joules/ $\text{mm}^2$ ) and assayed after 14 or 24 hours and in H0-1 cells treated for 24 hours with IFN- $\alpha$  + MEZ (2,000 units/ml + 10 ng/ml), or vincristine (0.1  $\mu\text{g/ml}$ ; 24 hours).

*mda-6* is a novel gene which displays the following properties: 1) its expression is increased during terminal differentiation (treatment with IFN- $\beta$  + MEZ for 96 hours); 2) treatment for 24 hours with IFN- $\beta$  + MEZ results in variable increases in its expression in all human melanomas tested and in an SV40-immortalized human melanocyte; 3) it is expressed in early stage melanomas (radial and early vertical growth phase melanomas), but not or at reduced levels in more advanced melanomas (metastatic melanomas); 4) it is expressed de novo and highly inducible by IFN- $\beta$  + MEZ within 24 hours in normal cerebellum; 5) it is not expressed de novo in GBM and only marginally induced in GBM after 24 hour treatment

-221-

with IFN- $\beta$  + MEZ; 6) high levels of de novo expression are seen in normal skin fibroblasts, colorectal carcinoma (SW613) and prostate carcinoma (LNCaP), but IFN- $\beta$  + MEZ treatment does not significantly alter expression; 7) endometrial adenocarcinoma (HTB113) cells display low levels of expression of this gene, whereas IFN- $\beta$  + MEZ treatment for 24 hours results in high levels of expression; 8) expression is increased in H0-1 cells treated with actinomycin D, adriamycin and VP-16; 9) expression is reduced in H0-1 cells treated with phenyl butyrate, gamma irradiation, cis-platinum and TNF- $\alpha$ ; and 10) expression is highest in normal melanocytes and a dysplastic nevus and reduced in radial growth phase (RGP) and vertical growth phase (VGP) primary melanomas and lowest in metastatic melanoma; 11) expression is reduced as a function of tumorigenic progression in Matrigel-progressed RGP and early VGP primary melanoma: 12) expression is low in tumorigenic and metastatic C8161 human melanoma cells and increased in three independent C8161 clones containing an inserted normal chromosome 6 that are tumorigenic but not metastatic: 13) an immediate early response gene i.e., induced in the presence of cycloheximide, in human promyelocytic leukemia. HL-60 cells induced to differentiate into monocytes and macrophages (treatment with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or vitamin D3) or granulocytes (treatment with all-trans retinoic acid (RA) or dimethyl sulfoxide (DMSO)); 14) induced as a function of growth arrest and differentiation in human neuroblastoma cells by treatment with the combination of phenylacetate and RA; and 15) induced during the induction of differentiation and growth arrest in human histiocytic lymphoma, U-937, cells by treatment with TPA.

mda-6 represents a terminal differentiation-regulated

-222-

gene displaying increased expression in all melanomas tested, in specific carcinomas, in normal cerebellum cells and in GBM cells treated with IFN- $\beta$  + MEZ. *mda-6* is also induced during the induction of monocyte/macrophage and granulocyte differentiation in human promyelocytic leukemia (HL-60) cells; differentiation in human neuroblastoma cells; and differentiation in histiocytic lymphoma (U-937) cells. This gene also displays increased expression in cells treated with specific chemotherapeutic agents, including adriamycin and VP-16. In contrast, expression of *mda-6* is decreased following treatment with gamma irradiation, the demethylating anticancer agent phenyl butyrate, the cytokine TNF- $\alpha$  and the chemotherapeutic agent cis-platinum. *mda-6* may be useful: 1) as a marker for specific tissue lineages and for distinguishing tumors of similar histotype (i.e., carcinomas, astrocytomas) (diagnostic applications); 2) to monitor response to topoisomerase inhibitors such as VP-16 and specific chemotherapeutic agents which function in a similar manner as adriamycin and cis-platinum (drug screening programs to identify new chemotherapeutic agents); 3) to identify compounds which have the capacity to induce terminal differentiation in human melanoma cells myeloid leukemic cells, histiocytic lymphoma and neuroblastoma (drug screening programs to identify new differentiation-inducing and chemotherapeutic agents); and 4) to monitor states of tumor progression, i.e., only expressed or expressed at higher levels in less aggressive and early stage cancers. Once full-length cDNAs are isolated, this gene (used in a sense orientation in an appropriate expression vector) may also prove useful in inhibiting growth and inducing terminal differentiation in human melanomas and other classes of tumors (therapeutic applications). Overexpression of this gene in specific

-223-

cell types (such as bone marrow cells) may also result in a decreased sensitivity of these cells to various DNA damaging agents and chemotherapeutic agents (therapeutic applications). This could prove useful in protecting bone marrow cells from damage induced by radiation and chemotherapy (therapeutic applications). Similarly, use of antisense constructs may also result in a decreased sensitivity to growth suppression in normal cells induced by specific classes of DNA damaging and therapeutic agents (therapeutic applications). This gene may also prove useful in the classification of more advanced astrocytomas (such as GBM) from less advanced earlier stages of astrocytomas (diagnostic applications). This gene may also prove useful in distinguishing between early stage (early radial growth phase, early vertical growth phase) melanoma and late stage (late vertical growth phase, metastatic) melanoma (diagnostic applications).

20 mda-6

ATGCCACGTGGGCTCATATGGGGCTGGGAGTAGTTGTCTTTCCTGGCACTAACGT  
TGAGCCCCCTGGAGGCACTGAAGTGCTTAGTGTAAGTATTGGGGTCTGACC  
CAAACACCTTCCAGCTCCTGTAACTACTGGCCTGGACTGTTTTCTCTCGCGCCT  
25 CCCCATGTGCTCCTGGTTCCCGTTTCCTCCACCTAGACTGTAAACCTCTCGCA  
(SEQ ID No. 8)

CCTGCAGTCCTGGAAGCGCGAGGGCCTCAAACGCGCTCTACATCTTCTGCCTTAG  
TCTCAGTTTGCGTGTCTTAATTATTATTGTTGTTTTAATTTAAACACCTCCTCAT  
30 GTACATACCCTGGCCGCCCCCTGCCCCCAGCCTCTCGGATTAGAATTATTTAAA  
CAAAACTAGGCGGTTGAATGAGAGGTTCTATGAGTACTGGGCATTTTTATTTT  
ATGAAATACTATTTAAAGCCTCCTCATCCCATGTTCTCCTTTTCCTCTCTCCCGG  
AGTT (Seq. ID No. 9)

35 Properties of mda-7

-224-

*mda-7* is a novel cDNA (it has no sequence homology with previously reported genes in the various DNA data bases).

Expression in H0-1 Human Melanoma Cells

5

Increased expression of *mda-7* after 24 hour treatment of H0-1 cells with recombinant human fibroblast interferon (IFN- $\beta$ ) (2000 units/ml), MEZ (10 ng/ml) and to the greatest extent with IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml).

10

Increased expression of *mda-7* is observed in H0-1 cells treated for 96 hours with IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml), MPA (3  $\mu$ M), IFN- $\beta$  + IFN- $\gamma$  (1000 units/ml + 1000 units/ml), IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml), MPA + MEZ (3  $\mu$ M + 10 ng/ml) and RA + MEZ (2.5  $\mu$ M + 10 ng/ml). Maximum induction is observed with IFN- $\beta$  + MEZ followed by MPA + MEZ and IFN- $\beta$  + IFN- $\gamma$ .

15

The relative level of *mda-7* induction correlates with the degree of growth suppression observed H0-1 cells treated with the various growth and differentiation modulating agents. The greatest increase in expression is observed in cells induced to irreversibly lose proliferative capacity and become terminally differentiated by treatment with IFN- $\beta$  + MEZ.

20

Expression in Additional Human Melanoma Cells

Increased expression of *mda-7* occurs in H0-1, C8161, C8161/6.3 (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells they are non-metastatic), F0-1, L0-1, SH-1, WM278 and WM239 human melanoma cells treated with IFN- $\beta$  + MEZ for

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-225-

24 hours. This gene is constitutively expressed in immortalized human melanocytes FM5169 (transformed by SV40). However, no increase in expression is observed in FM5169 following IFN- $\beta$  + MEZ treatment for 24 hours.

5

*mda-7* is either variably expressed or variably induced in all human melanoma cells treated with IFN- $\beta$  + MEZ. In contrast, although this gene is expressed in melanocytes, no change in expression is observed following a 24 hour treatment with IFN- $\beta$  + MEZ.

10

Expression in Normal Cerebellum, a Central Nervous System Tumor (Glioblastoma Multiforme) (GBM) and Normal Skin Fibroblast Cell Lines

15

*mda-7* is not expressed de novo in normal cerebellum, GBM or normal skin fibroblasts.

20

Expression of *mda-7* is induced in normal cerebellum, GBM and normal skin fibroblasts following a 24 hour treatment with IFN- $\beta$  + MEZ.

25

*mda-7* is not expressed de novo but is susceptible to induction by IFN- $\beta$  + MEZ in human cerebellum, GBM and normal human skin fibroblasts.

Expression in Colorectal Carcinoma (SW613), Endometrial Adenocarcinoma (HTB113) and Prostate Carcinoma (LNCaP)

30

*mda-7* is not expressed de novo in colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) or prostate carcinoma (LNCaP).

35

*mda-7* is not induced in colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) or prostate carcinoma

-226-

(LNCaP) cells following a 24 hour treatment with IFN- $\beta$  + MEZ.

5 This gene is neither expressed de novo or inducible by IFN- $\beta$  + MEZ in human carcinomas.

Effect of Various Treatment Protocols on Expression in H0-1 Cells

10 Treatment with IFN- $\beta$  (2000 units/ml; 24 hours), MEZ (10 ng/ml; 24 hours), IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml; 24 hours and 96 hours), IFN- $\alpha$  + MEZ (2000 units/ml + 10 ng/ml; 24 hours), adriamycin (0.1  $\mu$ g/ml; 24 hours), vincristine (0.1  $\mu$ g/ml; 24 hours), and UV (10 joules/mm<sup>2</sup>  
15 and assayed 24 hours later) results in increased *mda-7* expression in H0-1 cells. *mda-7* is also induced after 96 hour treatment with MPA (3  $\mu$ M), IFN- $\beta$  + IFN- $\gamma$  (1000 units/ml + 1000 units/ml), MPA + MEZ (3  $\mu$ M + 10 ng/ml) and RA + MEZ (2.5  $\mu$ M + 10 ng/ml). Highest level of  
20 expression observed in H0-1 cells treated with IFN- $\beta$  + MEZ for 24 or 96 hours.

No induction in *mda-7* expression is observed in H0-1 cells treated with IFN- $\alpha$  (2000 units/ml; 24 hours), IFN- $\gamma$   
25 (2000 unit/ml; 96 hours), phenyl butyrate (4 mM PB for 24 hours, 4 days or 7 days), cis-platinum (0.1  $\mu$ g/ml; 24 hours), gamma irradiation (treated with 3 gray and analyzed after 24 hours), actinomycin D (5  $\mu$ g/ml for 2 hours, assayed 24 hours later), TNF- $\alpha$  (100 units/ml; 24  
30 hours) or VP-16 (5  $\mu$ g/ml; 24 hours).

*mda-7* is a growth and differentiation and senescence-regulated novel gene which displays the following properties: 1) it is inducible during terminal

-227-

differentiation (treatment with IFN- $\beta$  + MEZ for 96 hours) and following treatment for 96 hours with many growth modulating and differentiation inducing agents; 2) treatment for 24 hours with IFN- $\beta$  + MEZ results in increased expression in all human melanomas tested, but not in an SV40-immortalized human melanocyte; 3) it is not expressed de novo but it is highly inducible by IFN- $\beta$  + MEZ within 24 hours in normal cerebellum, GBM and normal skin fibroblast cells; 4) it is not expressed or inducible in colorectal, endometrial or prostate carcinomas; 5) increased expression is induced in H0-1 cells treated with adriamycin, vincristine and UV irradiation; and 6) it is not expressed in growing human neuroblastoma cells but it is inducible following growth suppression and the induction of terminal differentiation; 7) it is not expressed in human promyelocytic leukemia (HL-60 and human histiocytic lymphoma (U-937) cells but it is induced following the induction of growth arrest and terminal differentiation; and 8) it is not expressed in actively growing human cells but it is induced during cellular senescence.

*mda-7* is a novel growth- and terminal differentiation-regulatable gene displaying increased expression in all melanomas (but not in melanocytes), and in normal skin fibroblasts and in both normal cerebellum and GBM cells treated with IFN- $\beta$  + MEZ. In contrast, *mda-7* is not expressed or induced in a series of carcinomas. *mda-7* may be useful: 1) as a marker for specific tissue lineage's (i.e., melanomas from keratinocytes) (diagnostic applications); 2) in distinguishing fibroblasts (inducible with IFN- $\beta$  + MEZ) from carcinomas (non-inducible with IFN- $\beta$  + MEZ) (diagnostic applications); 3) for the identification of agents capable of inducing growth suppression and various

-228-

components of the differentiation process (including terminal differentiation) in human melanomas (drug screening programs to identify new differentiation-inducing and chemotherapeutic agents); and 4) distinguishing melanocytes, and perhaps nevi, from early and late stage melanoma cells (diagnostic applications). Once full-length cDNAs are isolated, this gene (used in a sense orientation in an appropriate expression vector) may also prove useful in inhibiting growth and inducing terminal differentiation in human melanomas (therapeutic applications).

#### mda-7

CAGAATATTGTGCCCCATGCTTCTTTACCCCTCACAATCCTTGCCACAGTGTGGG  
CAGTGGATGGGTGCTTAGTAAGTACTTAATAAACTGTGGTGCTTTTTTTGGCCTG  
TCTTTGGATTGTTAAAAAACAGAGAGGGATGCTTGGATGTAACTGAACTTCAGA  
GCATGAAATCACACTGTCTCTGATATCT (SEQ ID No. 10)

#### Properties of mda-8

*mda-8* is a novel cDNA (it has no sequence homology with previously reported genes in the various DNA data bases)

#### Expression in H0-1 Human Melanoma Cells

Increased expression of *mda-8* results in H0-1 cells after 24 hour treatment with the combination of IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml).

Analysis of terminally differentiated H0-1 cells, i.e., H0-1 cells treated with the combination of IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml) for 96 hours indicate continued increased expression of *mda-8*.

-229-

Treatment of H0-1 cells for 96 hours with immune interferon (IFN- $\gamma$ ) (2000 units/ml) or IFN- $\beta$  + IFN- $\gamma$  (1000 units/ml + 1000 units/ml) results in enhanced *mda-8* expression. The level of increased *mda-8* expression at 5 96 hour is similar in IFN- $\gamma$ , IFN- $\beta$  + IFN- $\gamma$  and IFN- $\beta$  + MEZ treated H0-1 cells. (Note: The combination of IFN- $\beta$  + IFN- $\gamma$  results in a similar degree of growth suppression at 96 hour in H0-1 cells as does IFN- $\beta$  + MEZ. However, growth expression is reversible with the combination of 10 interferons, whereas it is irreversible with the combination of IFN- $\beta$  + MEZ).

*mda-8* is a novel IFN- $\gamma$ -inducible gene which also displays increased expression during terminal cell differentiation 15 in H0-1 human melanoma cells. *mda-8* could prove useful as a marker for immune interferon response and a marker for terminal differentiation in human melanoma cells.

#### Expression in Additional Human Melanoma Cells

20

Increased expression of *mda-8* occurs in H0-1, C8161 and WM278 human melanoma cells treated for 24 hours with IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml).

25 No change in expression of *mda-8* is seen in additional human melanomas treated for 24 hours with IFN- $\beta$  + MEZ, including C8161/6.3 (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental 30 C8161 cells they are non-metastatic), F0-1, L0-1, SH-1, and WM239.

Expression of this gene is increased by IFN- $\beta$  + MEZ in specific human melanoma cells.

35

-230-

Expression in Normal Cerebellum, a Central Nervous System Tumor (Glioblastoma Multiforme) (GBM) and Normal Skin Fibroblast Cell Lines

5     *mda-8* is expressed de novo in normal cerebellum, but not in GBM.

*mda-8* is expressed de novo in normal skin fibroblasts.

10    Growth for 24 hours in IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml) results in marginal changes in *mda-8* expression in normal cerebellum and normal skin fibroblasts.

15    Expression of *mda-8* is induced at high levels in GBM cells following a 24 hour exposure to IFN- $\beta$  + MEZ.

20    This gene is expressed de novo in both normal cerebellum and normal skin fibroblasts, but not in GBM. This gene is induced by IFN- $\beta$  + MEZ in human GBM, but expression is not altered in normal cerebellum cells and normal skin fibroblasts.

Expression in Colorectal Carcinoma (SW613), Endometrial Adenocarcinoma (HTB113) and Prostate Carcinoma (LNCaP)

25    *mda-8* is expressed de novo in colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) and prostate carcinoma (LNCaP).

30    Following a 24 hour treatment with IFN- $\beta$  + MEZ, expression of *mda-8* is unaffected in colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) and prostate carcinoma (LNCaP) cells.

35    *mda-8* is expressed de novo in the three types of

-231-

carcinomas. *mda-8* gene expression is not altered in the three carcinomas after treatment for 24 hours with IFN- $\beta$  + MEZ.

5     Effect of Various Treatment Protocols on Expression in H0-1 Cells

Increased expression of *mda-8* results after treatment with IFN- $\beta$  (2000 units/ml; 24 hours), actinomycin D (5  
10     $\mu$ g/ml for 2 hours, assayed 24 hours later), adriamycin (0.1  $\mu$ g/ml; 24 hours), cis-platinum (0.1  $\mu$ g/ml; 24 hours) and UV (10 joules/mm<sup>2</sup>, assayed 2, 14 and 24 hours later).

A 96 hour treatment with IFN- $\gamma$  (2000 units/ml), IFN- $\beta$  +  
15    MEZ (2000 units/ml) and IFN- $\beta$  + IFN- $\gamma$  (1000 units + 1000 units) results in increased *mda-8* expression.

No change in expression of *mda-8* is observed in H0-1 cells treated with MEZ (10 ng/ml; 24 or 96 hours), IFN- $\beta$   
20    (2000 units/ml; 24 or 96 hours), MPA (3  $\mu$ M; 96 hours), RA (2.5  $\mu$ M; 96 hours), MPA + MEZ (3  $\mu$ M + 10 ng/ml; 96 hours), RA + MEZ (2.5  $\mu$ M + 10 ng/ml), phenyl butyrate (4 mM PB for 24 hours, 4 days or 7 days), gamma irradiation (treated with 3 gray and analyzed after 24 hours),  
25    vincristine (0.1  $\mu$ g/ml; 24 hours), TNF- $\alpha$  (100 units/ml; 24 hours), VP-16 (5  $\mu$ g/ml; 24 hours), IFN- $\alpha$  (2000 units/ml) or IFN- $\alpha$  + MEZ (2000 units/ml + 10 ng/ml).

*mda-8* is a novel gene which displays the following  
30    properties: 1) it is inducible during terminal differentiation (treatment with IFN- $\beta$  + MEZ for 96 hours) and following treatment for 96 hours with recombinant gamma interferon (alone or in combination with IFN- $\beta$ ); 2) treatment for 24 hours with IFN- $\beta$  + MEZ results in

-232-

increased expression in only select human melanomas; 3) it is expressed de novo in normal cerebellum, normal skin fibroblasts, colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) and prostate carcinoma (LNCaP), but not in GBM; 4) treatment with IFN- $\beta$  + MEZ for 24 hours results in no change in expression in normal cerebellum, normal skin fibroblasts, colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) and prostate carcinoma (LNCaP); 5) treatment for 24 hours with IFN- $\beta$  + MEZ induces expression in GBM cells; and 6) increased expression is induced in H0-1 cells treated with actinomycin D, adriamycin, cis-platinum and UV irradiation.

*mda-8* is a cytokine- and terminal differentiation-responsive gene displaying increased expression in specific human melanomas and GBM cells treated with IFN- $\beta$  + MEZ (also inducible in H0-1 after 96 hour treatment with IFN- $\gamma$  and IFN- $\gamma$  + IFN- $\beta$ ). Enhanced expression is also apparent in H0-1 human melanoma cells treated with the transcription inhibitor actinomycin D, the chemotherapeutic agents adriamycin and cis-platinum and UV irradiation. *mda-8* may be useful: 1) as a marker for distinguishing between normal glial cells and malignant astrocytomas (such as GBM) (diagnostic applications); 2) to monitor response to type II interferon treatment (diagnostic applications); and 3) to identify compounds which have the capacity to induce terminal differentiation, induce similar cytotoxic effects as adriamycin, cis-platinum and UV irradiation (drug screening programs to identify new differentiation-inducing and chemotherapeutic agents). Once full-length cDNAs are isolated, this gene (used in a sense orientation in an appropriate expression vector) may prove useful in inhibiting growth and inducing terminal



-233-

differentiation in specific human melanomas and glioblastoma multiforme tumors (therapeutic applications).

5 mda-8

TTAAAGTTTGCCCTTGTGCTAAAGTGCCAGTGTATGTATGTTATACTTGATTG  
TTGTAAACTATATTTCAAAGTAAACCCTAGTGTATAATAAGTTTTATAACTAAAAAG  
GTTTAAGCTGCTAAAACTATTTTAAAGAGATGTGAAATCGAGTATGGGACTATCT  
10 TTTTTCCTCCTCTAAA (SEQ ID No. 11)

Properties of mda-9

mda-9 is a novel cDNA (it displays sequence homology to  
15 human transforming growth factor- $\beta$  (TGF- $\beta$ ) mRNA, 55.1%  
homology in 138 bp; GB-Pr:Humtgfb).

Expression in H0-1 Human Melanoma Cells

20 Increased expression of mda-9 occurs after 24 hour  
treatment of H0-1 cells with the combination of IFN- $\beta$  +  
MEZ (2000 units/ml + 10 ng/ml).

Increased expression of mda-9 persists in terminally  
25 differentiated H0-1 cells, i.e., H0-1 cells treated with  
the combination of IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml)  
for 96 hours.

mda-9 is a novel gene (with homology to TGF- $\beta$ ) which  
30 displays increased expression in terminally  
differentiated H0-1 human melanoma cells.

Expression in Additional Human Melanoma Cells

35 Variable increases in expression of mda-9 occurs in H0-1

-234-

and C8161 human melanoma cells treated for 24 hours with IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml).

5 The level of expression of *mda-9* decreases in SH-1 cells treated for 24 hours with IFN- $\beta$  + MEZ (2000 units/ml + 10  $\mu$ g/ml).

10 No change in *mda-9* expression results in FO-1, LO-1 or C8161/6.3 cells (a C8161 human melanoma cell clone containing an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells they are non-metastatic).

15 Expression of *mda-9* is increased by IFN- $\beta$  + MEZ in specific human melanoma cells. The lack of enhanced expression in C8161/6.3 cells treated with IFN- $\beta$  + MEZ, whereas parental C8161 cells do show an increase, suggests that modulation of this gene may correlate with more advanced stages of melanoma development (i.e.,  
20 melanoma cells with metastatic potential).

Effect of Various Treatment Protocols on Expression in H0-1 Cells

25 Increased expression of *mda-9* is observed in H0-1 cells treated with IFN- $\beta$  (2000 units/ml; 24 hours), MEZ (10 ng/ml; 24 hours), IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml; 24 and 96 hours), phenyl butyrate (4 mM PB for 24 hours, 4 days or 7 days), gamma irradiation (treated with 3 gray  
30 and analyzed after 24 hours), TNF- $\alpha$  (100 units/ml; 24 hours), IFN- $\alpha$  (2000 units/ml), IFN- $\alpha$  + MEZ (200 units/ml + 10 ng/ml), VP-16 (5  $\mu$ g/ml; 24 hours) or UV (10 joules/mm<sup>2</sup>, assayed 2 or 14 hours later).

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-235-

No change in *mda-9* expression is observed in H0-1 cells treated with actinomycin D (5  $\mu$ g/ml for 2 hours, assayed 24 hours later), UV (10 joules/mm<sup>2</sup>, assayed 24 hours later), cis-platinum (0.1  $\mu$ g/ml; 24 hours), vincristine (0.1  $\mu$ g/ml; 24 hours), IFN- $\beta$  (2000 units/ml; 96 hours), IFN- $\gamma$  (2000 units/ml; 96 hours), MEZ (10 ng/ml; 96 hours), MPA (3  $\mu$ M; 96 hours), RA (2.5  $\mu$ M; 96 hours), IFN- $\beta$  + IFN- $\gamma$  (1000 units/ml + 1000 units/ml; 96 hours), MPA + MEZ (3  $\mu$ M + 10 ng/ml; 96 hours) or RA + MEZ (2.5  $\mu$ M + 10 ng/ml).

*mda-9* is a novel gene with sequence homology to TGF- $\beta$  which displays the following properties: 1) it is inducible during terminal differentiation (treatment with IFN- $\beta$  + MEZ for 96 hours) in H0-1 human melanoma cells; 2) treatment for 24 hours with IFN- $\beta$  + MEZ results in increased expression in several human melanomas; 3) treatment for 24 hours with IFN- $\beta$  + MEZ results in increased expression in the tumorigenic and metastatic human melanoma C8161, but not in C8161/6.3 which is tumorigenic but not metastatic; and 4) increased expression is induced in H0-1 cells treated with a number of agents including phenyl butyrate, gamma irradiation, TNF- $\alpha$ , UV irradiation (after 2 and 14 hours, but not after 24 hours), IFN- $\alpha$  and IFN- $\alpha$  + MEZ.

*mda-9* is a terminal differentiation-responsive gene displaying increased expression in several human melanomas treated with IFN- $\beta$  + MEZ. Enhanced expression is induced by IFN- $\beta$  + MEZ in the tumorigenic and metastatic human melanoma cell C8161, but not its reverted derivative C8161/6.3 (which retains tumorigenicity, but has lost metastatic potential). Increased expression is also apparent in H0-1 human

-236-

melanoma cells treated with the demethylating anticancer agent phenyl butyrate, the cytokine TNF- $\alpha$ , gamma irradiation and UV irradiation.

5     *mda-9* may be useful: 1) as a marker for distinguishing  
between early stage and more progressed human melanoma  
(diagnostic applications); and 2) to identify compounds  
which have the capacity to induce terminal  
10     differentiation and to induce specific patterns of DNA  
damage as induced by UV irradiation and gamma irradiation  
(drug screening programs to identify new differentiation-  
inducing and chemotherapeutic agents). Once full-length  
cDNAs are isolated, this gene (used in a sense  
15     orientation in an appropriate expression vector) may also  
prove useful in inhibiting growth and inducing terminal  
differentiation in specific human melanomas (therapeutic  
applications). When used in an antisense orientation,  
expression of this gene might allow normal cells (such as  
bone marrow cells) to be engineered to be resistant to  
20     cytotoxicity induced by specific chemotherapeutic agents  
and gamma irradiation (therapeutic applications).

*mda-9*

25     AAAACTTTCAAGAGATTTACTGACTTTTCCTAGAATAGTTTCTCTACTGGAAACCT  
GATGCTTTTATAAGCCATTGTGATTAGGATGACTGTTACAGGCTTAGCTTTGTGT  
GAAAACCAGTCACCTTTCTCCTAGGTAATGAGTAGTGCTGTTTCATATTACTTTAG  
TTCTATAGCATACTCGATCTTTAACATGCTATCATAGTACATTAGATGATG (SEQ  
ID No. 12)

30

Additional *mda* Genes Isolated Using Subtraction  
Hybridization from H0-1 Human Melanoma Cells Treated with  
IFN- $\beta$  + MEZ

35     *mda-1*: Novel gene which displays increased expression in

-237-

IFN- $\beta$  and IFN- $\beta$  + MEZ treated H0-1 cells after 24 hours (HP 2-36). (Jiang and Fisher, Molecular and Cellular Differentiation, 1 (3), in press, 1993).

5     *mda-2*: Novel gene which displays increased expression in IFN- $\beta$  and IFN- $\beta$  + MEZ treated H0-1 cells after 24 hours (HP-3-31). (Jiang and Fisher, Molecular and Cellular Differentiation, 1 (3), in press, 1993).

10    *mda-3*: Increased expression in MEZ and IFN- $\beta$  + MEZ treated H0-1 cells after 24 hours (HP 2-4). (Identical to Human GOS 19-1 mRNA, cytokine (Gb-Pr:Hummpla), human TPA-inducible mRNA, pLD78 (GB-Pr:Humpld78). (Jiang and Fisher, Molecular and Cellular Differentiation, 1 (3), in  
15    press, 1993).

*mda-11*: Novel gene which displays increased expression in IFN- $\beta$  + MEZ treated H0-1 cells after 24 hours. (HJ 2-78). (87.2% identity to the rat ribosomal protein  
20    IF116).

CGCACGTCACCCACCTTCCGGCGGCCGAAGACACTGCGACTCCGGAGACAGCCCA  
AATATCCTCGGAAGAGCGCTCCCAGGAGAAACAAGCTTGACCACTATGCTATCAT  
CAAGTTTCCGCTGACCACTGAGTCTGCCATGAAGAAGATAGAAGACAACAACACA  
25    CTTGTGTTCATTGTGGATGTTAAAGCCAACAAGCACCAGATTAACAGCTGTGAGA  
GCTGTATGACATTGATGTGCAGTACACCTGATCGTCT (Seq. ID No. 13)

*mda-12*: Gene which displays increased expression in IFN- $\beta$  + MEZ treated H0-1 cells after 24 hours. (HP 3-8).  
30    (Identical to Human GOS19-3 mRNA (Gb-Humcpgcus2), LD78A (Gb-Pr:Humld78a).

*mda-13*: Gene which displays increased expression in IFN- $\beta$  and IFN- $\beta$  + MEZ treated H0-1 cells after 24 hours. (HP  
35    S-7). (Identical to interferon stimulated gene -56

-238-

(ISG56), an IFN- $\beta$  inducible gene).

5 *mda-14*: Gene which displays increased expression in IFN- $\beta$  + MEZ treated H0-1 cells after 24 hours. (HP 2-59 and HP 3-114, same gene isolated independently two times). (Identical to interleukin-8 (IL-8) (Gb-Un:M28130), human mRNA for MDNCF (monocyte derived neutrophil chemotactic factor) (Gb-Pr:Nummdncf).

10 TAAAAAATTTCATTCTCTGTGGTATCCAAGAATCAGTGAAGATGCCAGTGAAACT  
TCAAGCAAATCTACTTCAACACTTCATGTATTGTGTGGGTCTGTTGTAGGGTTGC  
CAGATGCAATACAAGATTCCTGGTTAAATTTGAATTTTCAGTAAACAATGAATAGT  
TTTTCATTGTACATGAAATATCAGAACATACTTATATGTAAGTATATTATTGATG  
ACAAACACAATATTTAATATA (Seq. ID No. 14)

15 *mda-15*: Gene which displays increased expression in IFN- $\beta$  + MEZ treated H0-1 cells after 24 hours (HP 2-64). (Identical to vimentin, intermediate filament protein (Gb-Pr:Humviment).

20 *mda-16*: Gene which displays increased expression in IFN- $\beta$  + MEZ treated H0-1 cells after 24 hours. (HP 2-18). (Identical to human apoferritin H gene (Gb-Pr:Humferg2).

25 *mda-17*: Gene which displays increased expression in IFN- $\beta$  + MEZ treated H0-1 cells after 24 hours. (HP 2-40). (Identical to IFP-53 (Gb-Pr:Humifp), IFN-inducible gamma 2 protein (Gb-Huminfig).

30 GGGGGTGAAACTTTCCAGTTTACTGAACTCCAGACCATGCATGTAGTCCACTCCA  
GAAATCATGCTCGCTTCCTTGGCACACAGTGTTCTCCTGCCAAATGACCCTAGAC  
CCTCTGTCCTGCAGAGTCAGGGTGGCTTTACCCTGACTGTGTTCGATGCAGAGTC  
TGCTCGACAGAT (Seq. ID No. 15)

35 *mda-18*: Gene which displays increased expression to IFN- $\beta$

-239-

+ MEZ treated H9-1 cells after 24 hours (HP 2-45).  
(Identical to hnRNP A1 protein (Gb-Pr:Humrnpa1), RNA  
binding protein (Gb-Pr:Humhnrnpa)).

5 TACGATCAGACTGTTACATTTAGCAATCAACAGCATGGGGCGAAAAAAAAAATC  
TACTTAAAACCCTTTGTTGGAATGCTTTACACTTTCCACAGAACAGAACTAAAA  
TAACTGTTTACATTAGTCACAATACAGTCTCGA (Seq. ID No. 16)

-240-

Fifth Series of Experiments

The carcinogenic process often proceeds through a series of interrelated stages and is regulated by multiple genetic changes and environmental factors (1-6). Although the specific events controlling each component of this multistep process remain to be defined, a recurrent theme in many cancer cells is an aberrant pattern of differentiation (7-10). In addition, as cancer cells evolve, ultimately developing new phenotypes or an increased expression of pre-existing transformation-related phenotypes, the degree of expression of differentiation associated traits is often further diminished. Malignant melanoma epitomizes the process of tumor progression and emphasizes the selective nature of the metastatic phenotype and the growth dominant properties of metastatic cells (11-14). Of the numerous types of cancer developing in North American populations, melanoma is increasing at the fastest rate and it is estimated that as many as 1 in 100 currently born children may eventually develop superficial spreading type melanoma (11). Although melanoma is readily curable at early stages, surgical and chemotherapeutic interventions are virtually ineffective in preventing metastatic disease and death in patients with advanced stages of malignant melanoma. These observations emphasize the need for improved therapeutic approaches to more efficaciously treat patients with metastatic melanoma.

Development of malignant melanoma in humans, with the exception of nodular type melanoma, consists of a series of sequential alterations in the evolving tumor cells (11-15). These include conversion of a normal melanocyte into a common acquired melanocytic nevus (mole),



-241-

followed by the development of a dysplastic nevus, a radial growth phase (RGP) primary melanoma, a vertical growth phase (VGP) primary melanoma and ultimately a metastatic melanoma (Figure 20). Although readily  
5 treatable during the early stages of development, even during the VGP if the lesion is  $\leq$  0.76-mm thick, currently employed techniques are not very effective (<20% survival) in preventing metastatic spread and morbidity in patients with VGP lesions  $>$  4.0-mm thickness  
10 (11). This exceptional model system is ideally suited to evaluate the critical gene expression changes that mediate both the early and late phases of melanoma evolution.

15 A potentially less toxic approach to cancer therapy involves a process termed differentiation therapy (7,9,10,16,17). Two premises underlie this therapeutic modality. (A) Many types of neoplastic cells display aberrant patterns of differentiation resulting in  
20 unrestrained growth; and (B) Treatment with the appropriate agent(s) can result in the reprogramming of tumor cells to lose proliferative capacity and become terminally differentiated. Intrinsic in this hypothesis is the assumption that the genes that mediate normal  
25 differentiation in many tumor cells are not genetically defective, but rather they fail to be expressed appropriately. The successful application of differentiation therapy in specific instances may result because the appropriate genes inducing the differentiated  
30 phenotype become transcriptionally activated resulting in the production of necessary gene products required to induce terminal cell differentiation. Applicants have tested this hypothesis using human melanoma cells  
(8,10,18-23). Treatment of human melanoma cells with the  
35 combination of recombinant human fibroblast interferon

-242-

(IFN- $\beta$ ) and the antileukemic compound mezerein (MEZ) results in a rapid cessation of growth, an induction of morphological changes, an alteration in antigenic phenotype, an increase in melanin synthesis and an irreversible loss in proliferative capacity, i.e., terminal cell differentiation (18,21,23). In contrast, treatment of the same melanoma cells with equivalent doses of either IFN- $\beta$  or MEZ alone results in specific differentiation-related and immunologically-related changes and growth suppression, but terminal differentiation does not occur (18,21,24-34).

This invention summarizes the results of applicants' analysis of the process of reversible and irreversible (terminal) differentiation in human melanoma cells. By using the technique of subtraction hybridization a series of novel genes, termed melanoma differentiation associated (*mda*), have been identified that display enhanced expression during differentiation and growth arrest in human melanoma cells. These newly identified *mda* genes should prove useful in defining the molecular basis of human melanoma growth, differentiation and transformation progression.

Dissecting the Processes of Growth Control and Differentiation in Human Melanoma Cells

The process of terminal differentiation in H0-1 cells involves a number of changes in cellular phenotype and gene expression (18,21,23,35). Biochemical and cellular changes include growth suppression, changes in melanin synthesis (biochemical differentiation) and modified antigenic properties (immunologic differentiation) (18,21,23,29,34,35). The ability to define the relationship between the different components of growth

-243-

and differentiation and the corresponding gene expression changes induced in human melanoma cells has been assisted by the identification of specific compounds that induce different components of these processes (Figure 21A-H)

5 (18,21,23,33,36).

Growth of H0-1 cells in the combination of IFN- $\beta$  plus recombinant immune interferon (IFN- $\gamma$ ) results in a similar level of growth suppression after 96 hr as does

10 the combination of IFN- $\beta$  + MEZ (23,33). However, this combination of interferons induces reversible growth arrest and it does not cause an increase in melanin synthesis above that induced by IFN- $\beta$  alone (33). This specific combination of agents permits a dissociation

15 between reversible growth suppression and induction of irreversible growth suppression and terminal differentiation. Treatment of H0-1 cells with compounds such as all-trans retinoic acid (RA), mycophenolic acid (MPA), IFN- $\beta$  and MEZ results in a reversible increase in

20 melanin synthesis, i.e., reversible biochemical differentiation (Figure 21A-H) (23,36). However, although MPA, IFN- $\beta$  and MEZ induce growth suppression, RA increases melanin and tyrosinase levels without altering H0-1 growth (23,26]. These results indicate that RA can

25 be used to identify gene expression changes correlating directly with increased melanin synthesis in the absence of growth suppression. Additional compounds that have proven of interest, include MPA and MEZ that induce morphologic differentiation in H0-1 cells (Figure 22).

30 These changes in H0-1 cells are also reversible following growth in the absence of the inducing agent. The only currently available combination of agents that can irreversibly induce the spectrum of differentiation changes resulting in terminal cell differentiation in H0-

35 1 cells is IFN- $\beta$  + MEZ (18,21,23).

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-245-

Table 3. Gene Expression Changes Observed in Terminally Differentiated H0-1 Cells

	<u>Increased</u> <u>Expression</u>	<u>Decreased</u> <u>Expression</u>	<u>No</u> <u>Change in</u> <u>Expression</u>
5			
	<i>c-jun</i>	<i>c-myc</i>	<i>c-fos</i>
10	<i>jun-B</i>	cyclin A	RB
	HLA Class I	cyclin B	N-cadherin
15	ISG-15	tenascin	
	ISG-54	$\gamma$ -actin	
	gro/MGSA	$\beta$ -actin	
20	$\alpha_5$ integrin	cdc 2	
	$\beta_1$ integrin	histone H1	
25	fibronectin	histone H4	
30	H0-1 cells were grown for 96 hr in the presence of 2000 units/ml IFN- $\beta$ and 10 ng/ml MEZ prior to isolation of total RNA and analysis of gene expression by Northern blotting analysis (23). Cells remain viable under these conditions, but they irreversibly lose proliferative ability (18,23).		

-246-

Autocrine Factors Involved in Gene Expression Changes  
Induced in Human Melanoma Cells During Reversible Growth  
Suppression and Terminal Cell Differentiation

5

A potentially important mediator of growth arrest during differentiation in hematopoietic cells is autocrine IFN- $\beta$  (37-39). Evidence suggesting a link between autocrine IFN- $\beta$  and differentiation in hematopoietic cells include:

- 10 (a) the observation that IFN- $\beta$  neutralizing antibodies can partially abrogate the reduction in c-myc levels and growth suppression occurring during hematopoietic differentiation; (b) the induction of interferon regulatory factor 1 (IRF-1) during myeloid  
15 differentiation; (c) the partial reversal by IRF-1 antisense oligomers of growth inhibition and the induction of differentiation induced in leukemic cells by interleukin-6 and leukemia inhibitory factor; and (d) the  
20 induction of specific type I interferon (IFN- $\alpha/\beta$ ) gene expression during terminal differentiation in hematopoietic cells (37-39).

Enhanced expression of interferon responsive-genes and the gro/MGSA gene occurs in HO-1 cells during the  
25 processes of reversible and irreversible differentiation (23). These observations suggest that autocrine-feedback pathways could contribute to the gene expression changes observed during the differentiation process. To directly  
test for this possibility, HO-1 cells were treated with  
30 various inducing agents for 24 hr, cultures were washed free of inducers and then grown for 72 hr in medium lacking the inducing compounds. The conditioned medium from these cells was collected and tested for its ability  
to induce gene expression changes in naive HO-1 cells  
35 (23). Conditioned medium obtained from cells treated

-247-

with IFN- $\beta$  + MEZ induced growth suppression and a number of gene expression changes also apparent in inducer-treated H0-1 cells (23,40). These include, enhanced c-jun,  $\alpha$ , integrin and fibronectin expression and induction of jun-B, HLA Class I, ISG-15, and gro/MGSA expression (23). These observations suggest two autocrine loops may be associated with differentiation in H0-1 cells, one involving an autocrine IFN- $\beta$  and the other an autocrine gro/MGSA. Support for the IFN- $\beta$  autocrine loop is indicated by the ability of IFN- $\beta$  antibodies to partially neutralize ISG-15 induction by conditioned medium and the direct induction of the IFN- $\beta$  gene as monitored by RT-PCR by conditioned medium (40). However, conditioned medium from IFN- $\beta$  + MEZ-treated H0-1 cells does not induce terminal differentiation in H0-1 cells (40). Similarly, additional agents that induce reversible growth arrest and differentiation also produce conditioned medium that can induce type I interferon responsive genes in naive H0-1 cells. These results suggest that specific autocrine loops may also contribute to growth inhibition and the differentiation process in solid tumors such as human melanoma.

Identification of Genes Differentially Expressed During the Processes of Differentiation and Growth Suppression in Human Melanoma Cells

To directly identify genes displaying differential expression in human melanoma cells induced to terminally differentiate applicants have used a modified subtraction hybridization approach (Figure 8) (41). cDNA libraries were prepared from poly (A+) RNA obtained from untreated H0-1 cells (Ind $\bar{c}$  cDNA library; driver cDNA library), and H0-1 cells treated with IFN- $\beta$  + MEZ for 2, 4 8, 12 and 24

-248-

(*Ind*<sup>-</sup> cDNA library; tester cDNA library). Tester and driver cDNA libraries were directionally cloned into the commercially available  $\lambda$  Uni-ZAP phage vector. Subtraction hybridization was then performed between double-stranded tester DNA and single-stranded driver DNA prepared by mass excision of the libraries. The subtracted cDNAs were efficiently cloned into the  $\lambda$  Uni-ZAP phage vector, which permits easy manipulation for both screening and gene characterization. A single round of subtraction of untreated H0-1 control (*Ind*<sup>-</sup>) cDNAs from IFN- $\beta$  + MEZ-treated (*Ind*<sup>+</sup>) cDNAs resulted in the identification of a series of cDNAs displaying differential expression in untreated versus differentiation inducer-treated H0-1 cells. These cDNAs are referred to as melanoma differentiation associated (*mda*) cDNAs. Initially 70 cDNA clones were analyzed and 23 clones were found to display differences in gene expression between *Ind*<sup>-</sup>- and *Ind*<sup>+</sup>-treated H0-1 cells (41). As anticipated, subtraction of control H0-1 cDNAs from IFN- $\beta$  + MEZ-treated H0-1 cDNAs resulted in a series of MDA genes that displayed enhanced expression after 24-h treatment with the various inducers. These included genes displaying enhanced expression in H0-1 cells treated with both IFN- $\beta$  and IFN- $\beta$  + MEZ (*mda*-1 and *mda*-2), MEZ and IFN- $\beta$  + MEZ (*mda*-3), IFN- $\beta$ , MEZ and IFN- $\beta$  + MEZ (*mda*-4) and uniquely by IFN- $\beta$  + MEZ (*mda*-5 and *mda*-6) (Figure 10) (41). Of these six *mda* genes, only *mda*-3 originally represented a previously identified gene, GOS-19-1 (41). Analysis of eight additional human melanoma cell lines indicates that specific *mda* genes also display enhanced expression following a 24-h treatment with IFN- $\beta$  + MEZ (data not shown).

The studies described above indicate that specific *mda*



-249-

genes exhibit elevated expression in H0-1 cells after 24-h treatment with appropriate inducing agents. Studies were performed to monitor *mda* expression in H0-1 cells treated with IFN- $\beta$ , MEZ or IFN- $\beta$  + MEZ for 96-h (Figure 15). Additionally, the pattern of expression of specific *mda* genes under experimental conditions inducing reversible growth suppression (IFN- $\beta$  + IFN- $\gamma$ , IFN- $\beta$ , IFN- $\gamma$ , MEZ, MPA, MPA + MEZ, RA + MEZ), increased melanin synthesis (IFN- $\beta$ , MEZ, MPA, RA, IFN- $\beta$  + MEZ, MPA + MEZ, RA + MEZ), increased melanin synthesis (IFN- $\beta$ , MEZ, MPA, RA, IFN- $\beta$  + MEZ, MPA + MEZ, RA + MEZ), morphological changes (MPA, MEZ, IFN- $\beta$  + MEZ, MPA + MEZ, RA + MEZ) or terminal cell differentiation (IFN- $\beta$  + MEZ) was determined. These experiments indicate continuous elevated expression of *mda-4*, *mda-5*, *mda-6* (p21), *mda-7*, *mda-8* and *mda-9* in terminally differentiated H0-1 cells (Figure 15). A differential pattern of expression of the *mda* genes was observed in H0-1 cells treated with the various differentiation and growth modulating agents. Three of the *mda* cDNAs, *mda-4*, *mda-5* and *mda-8*, displayed overlapping induction profiles in H0-1 cells. These genes displayed elevated expression in H0-1 cells treated for 96 hr with IFN- $\beta$  + MEZ, IFN- $\gamma$  or IFN- $\beta$  + IFN- $\gamma$  (Figure 15). These cDNAs, which have not been previously described in any DNA data base, may correspond to new classes of cytokine-responsive genes. This possibility is currently under investigation. Expression of *mda-7* was increased in H0-1 cells treated for 96 h with agents inducing growth arrest, including IFN- $\beta$ , MEZ, MPA, IFN- $\beta$  + IFN- $\gamma$ , IFN- $\beta$  + MEZ, MPA + MEZ, RA + MEZ. The degree of increase in *mda-7* expression was greatest in H0-1 cells treated with IFN- $\beta$  + MEZ that also induces terminal cell differentiation. Treatment of H0-1 cells for 96 hr with RA does not induce growth changes or induce *mda-7* expression. Similarly, IFN- $\gamma$  that only marginally inhibits H0-1

-250-

growth also does not result in significant *mda-7* expression. In the case of *mda-9*, increased expression in H0-1 cells was only apparent in terminally differentiated cells treated with IFN- $\beta$  + MEZ (Figure 15). Further studies are in progress to isolate full-length cDNAs for the various novel *mda* genes and to analyze their expression during the processes of growth and differentiation in human melanoma and other human cell types.

10     The Melanoma Differentiation Associated Gene-6 (*mda-6*) is the Cyclin-Dependent Kinase Inhibitor, p21

Cell-cycle regulation results from the ordered activation of a series of related enzymes referred to as cyclin-dependent kinases (CDKs) (42). In normal cells, CDKs are predominantly found in multiple quaternary complexes, consisting of CDK, a cyclin, proliferating cell nuclear antigen (PCNA) and the p21 protein (43,44). p21 controls CDK activity, thereby affecting cell-cycle control and growth in mammalian cells (43-50). Using human glioblastoma cells containing an inducible wild-type p53 tumor suppressor gene and subtraction hybridization, a gene called WAF1 (wild-type p53-activated fragment 1) that encodes an M<sub>21,000</sub> protein was identified (49,50). WAF1 is the same p21-encoding gene identified using the two-hybrid system as a potent CDK inhibitor, referred to as CPI1 (Cdk-interacting protein 1) (46). p.21 levels have been shown to increase in senescent cells (gene referred to as sdi-1; senescent cell-derived inhibitor) (51) and overexpression of p21 inhibits the growth of tumor cells (46,49,51). Treatment of wild-type p53 containing cells with DNA damaging agents results in elevated wild-type p53 protein and increased p21 levels (51). In this context, p21 may directly contribute to G<sub>1</sub> growth arrest

-251-

and apoptosis resulting in specific target cells after induction of DNA damage (51). Recent studies also demonstrate that p21 can: directly inhibit PCNA-dependent DNA replication in the absence of a cyclin/CDK; and inhibit the ability of PCNA to activate DNA polymerase  $\delta$  by directly interacting with PCNA (52). These studies indicate that p21 is an important component of growth control, cell-cycle progression, DNA replication and the repair of damaged DNA.

10

Sequence analysis of *mda-6* indicates that it is the CDK inhibitor p21 (41) (Figure 23A + B; GenBank accession number U09579). The cloning of this gene from a differentiation-inducer treated human melanoma library, indicates that *mda-6* (p21) may contribute to the induction of growth arrest observed in terminally differentiated human melanoma cells. Like WAF1, *mda-6* is also induced in human melanoma cells following DNA damage resulting from treatment with methyl methanesulfonate (53). In human melanoma, *mda-6* expression is increased during terminal differentiation, rapidly induced by incubation in serum free medium and enhanced in cells grown to high saturation densities (53). Several lines of evidence indicate that the expression of *mda-6* inversely correlates with melanoma progression (53). These include: (A) the presence of higher levels of *mda-6* in actively growing melanocytes and nevi and reduced levels in radial and early vertical growth phase primary melanomas as well as metastatic human melanomas (53); (B) decreased expression of *mda-6* in early vertical growth phase primary human melanoma cells selected for autonomous or enhanced tumor formation in nude mice (53,54); and (C) increased levels of *mda-6* mRNA in metastatic human melanoma cells displaying a loss of metastatic potential resulting following introduction of a normal chromosome 6 (53,55). Taken

-252-

together, these recent studies indicate that p21 (mda-6/WAF1/CIP1/CAP20/sdi-1) may function as a negative regulator of melanoma growth, progression and metastasis.

5 CDK inhibitors, in addition to p21, have also been identified (56-59). These include: a 16-kDa protein, p16<sup>INK4</sup> (inhibitor of cyclin-dependent kinase 4), that specifically inhibits cyclin D/Cdk4 (56); a 27-kDa inhibitory protein, p27<sup>KIP1</sup> (kinase inhibitory protein 1),  
10 induced in transforming growth factor- $\beta$ -arrested and contact-inhibited cells (57,58); and a 28-kDa protein, p28<sup>ICK</sup> (inhibitor of cyclin-dependent kinase), that binds to and inhibits the kinase activity of preformed Cdk/cyclin complexes in human cells (59). It is not presently  
15 known if any or all of these CDK inhibitors contribute to the process of differentiation inducer-mediated growth arrest and terminal cell differentiation in human melanoma.

## 20 Summary and Perspectives

It is now possible to reprogram cultured human melanoma cells to an earlier stage in their development by treatment with the appropriate inducing agents. This process,  
25 an important component of differentiation therapy, can result in a rapid loss of proliferative potential and terminal differentiation in these cancer cells. By using the appropriate inducers, it is possible to manipulate specific components of the differentiation program in a  
30 reversible or irreversible (terminal cell differentiation) manner. This capability results in a powerful model system permitting the systematic dissection of the roles of specific genes and biochemical pathways in regulating growth, differentiation and oncogenic poten-

-253-

tial in human melanoma cells. The combination of IFN- $\beta$  + MEZ induces an irreversible loss of growth potential and terminal cell differentiation in human melanoma cells. At comparable concentrations, IFN- $\beta$  or MEZ alone  
5 induce certain components of the differentiation process, but they do not induce an irreversible loss of growth potential or terminal differentiation. The process of terminal differentiation in human melanoma cells treated with IFN- $\beta$  + MEZ involves specific biochemical, struc-  
10 tural, immunological and gene expression alterations.

To identify the critical gene expression changes associated with and controlling terminal differentiation in human melanoma cells applicants have used a modified  
15 subtraction hybridization protocol. This approach has resulted in the identification of a series of melanoma differentiation associated (*mda*) genes, including both previously identified and novel, that display elevated expression in human melanoma cells treated with differen-  
20 tiation and growth suppressing agents. One of the *mda* genes, *mda-6*, is identical to the cyclin-dependent kinase inhibitor p21 (also referred to as WAF1, CAP20, CIP1, and sdi-1). Initial studies using a panel of unique *mda* genes indicate that increased expression of specific *mda*  
25 genes' results following treatment with agents inducing growth arrest and terminal differentiation as well as defined classes of DNA damaging and chemotherapeutic agents (Figure 24). Specific *mda* genes also display differential expression as a function of human melanoma  
30 progression, in normal versus tumor-derived cells of neuroectodermal origins and in additional cell differentiation model systems.

Future studies using the *mda* genes should prove valuable  
35 in defining the molecular determinants mediating growth

-254-

control, tumor progression, response to chemotherapy and terminal cell differentiation in human melanoma and other tumors. The *mda* genes will also prove useful as part of a simple genetic screen for identifying and monitoring agents inducing specific DNA damage pathways and for identifying agents capable of inducing terminal differentiation in cancer cells. This information should prove important in developing improved therapeutic modalities for metastatic melanoma and for additional human malignancies.

-255-

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-265-

Sixth Series of Experiments

The multistep carcinogenic process is often characterized by discrete changes in cellular phenotype, including resistance to normal growth inhibitory controls and aberrant patterns of differentiation (Fisher & Rowley, 1991; Knudson, 1993; Hoffman & Liebermann, 1994; Jiang et al., 1994). Treatment of specific cancers with differentiation modulating agents can result in a suppression of growth and the induction of a more mature differentiated phenotype (Sachs, 1978; Jimenez & Yunis, 1987; Waxman et al., 1988, 1991; Fisher & Rowley, 1991; Lotan, 1993). The mechanism underlying these profound effects on cellular physiology are not currently known. In the case of human melanoma, the combination of recombinant fibroblast interferon (IFN- $\beta$ ) and the anti-leukemic compound mezerein (MEZ) results in an irreversible loss of proliferative capacity and terminal cell differentiation (Fisher et al., 1985; Jiang et al., 1993). This model system, combined with subtraction hybridization, is being used to define the molecular basis of growth control and cancer cell differentiation (Jiang & Fisher, 1993; Jiang et al., 1994). Using a differentiation-induction plus subtraction hybridization approach, a series of differentially expressed cDNAs, termed melanoma differentiation associated (mda) genes, have been identified that display enhanced expression as a function of growth suppression and terminal cell differentiation (Jiang & Fisher, 1993; Jiang et al., 1994).

The human p21 cyclin-dependent kinase (Cdk)-interacting protein CIP1 (Xiong et al., 1993b; Harper et al., 1993), and the mouse CAP20 homologue (Gu et al., 1993), is a ubiquitous inhibitor of cyclin kinases and an integral

-266-

component of cell cycle control. This gene is identical to the WAF1 (wild-type (wt) p53 activated factor-1) gene identified following induction by wt p53 protein expression in a human glioblastoma multiforme cell line (El-Deiry et al., 1993). p21 has also been independently cloned as a consequence of induction of senescence in normal human foreskin fibroblast cells, SDI1 (senescent cell-derived inhibitor-1) (Noda et al., 1994), and during the process of terminal cell differentiation in human melanoma cells, *mda-6* (Jiang and Fisher, 1993; Jiang et al., 1994). p21 is a nuclear localized protein that is induced by DNA damage and during apoptosis in specific cell types as a function of wt p53 activation (El-Deiry et al., 1993, 1994). These studies suggest that p21 may be an important downstream mediator of wt p53-induced growth control in mammalian cells (El-Deiry et al., 1993, 1994). Somewhat paradoxical data indicates that WAF1/CIP1 is induced as an immediate-early gene following mitogenic stimulation of growth arrested cells in a p53-independent manner (Michieli et al., 1994). Applicants presently demonstrate that *mda-6* (WAF1/CIP1/SDI1) expression is also induced by mechanistically diverse acting agents resulting in macrophage/monocyte (TPA and Vit D3) or granulocyte (RA and DMSO) differentiation in human promyelocytic leukemia cells (Collins, 1987), HL-60, that lack endogenous p53 genes (Wolf and Rotter, 1985). Using differentiation-resistant variants (Homma et al., 1986; Mitchell et al., 1986), a direct correlation is found between the early induction of *mda-6* expression and the onset of specific programs of differentiation in HL-60 cells. Applicants' results indicate that sustained p21 expression can be maintained in the absence of wt p53 protein and elevated levels of p21 (WAF1/CIP1/SDI1) mRNA and protein correlate with growth suppression and differentiation induction in a

-267-

p53-independent manner in HL-60 cells.

### Experimental Results

5     Treatment with diverse acting differentiation inducing  
agents results in increased *mda-6* (WAF1/CIP1/SDI1)  
expression in HL-60 cells

10     HL-60 is a differentiation competent myeloid leukemia  
cell line that can be induced to differentiate along both  
monocytic and granulocytic lineages following exposure to  
the appropriate inducing agents (Gallagher et al., 1979;  
Huberman & Callahan, 1979; Breitman et al., 1980;  
Collins 1987). Treatment of HL-60 cells with TPA, that  
15     commit these cells to a macrophage-like lineage (Lotem &  
Sachs, 1979; Rovera et al., 1979), induces *mda-6*  
expression as detected by both Northern blotting (Fig.  
25A-B) and RT-PCR (Fig. 26A-C). Induced expression  
following TPA treatment (3 nM) occurs within 2 h of  
20     exposure and elevated *mda-6* levels persist in terminally  
differentiated HL-60 cells (Fig. 25A-B and 27A-C).  
Similarly, Vit D3 (400 nM), that also commits HL-60 cells  
to a monocyte-macrophage-like lineage (Miyaura et al.,  
1981; Tanaka et al., 1982), induces *mda-6* within 1 h of  
25     treatment and elevated expression continues in terminally  
differentiated HL-60 cells (Figs. 26A-C and 27A-C).  
Induction of a granulocyte-like phenotype in HL-60 cells  
by RA (Breitman et al., 1980) or DMSO (Collins et al.,  
1978) also induces *mda-6* mRNA production. In the case of  
30     RA (1  $\mu$ M), induction of *mda-6* is apparent within 3 h and  
expression remains elevated 6 days post-RA treatment  
(Figs. 25A-B, 26A-C and 27A-C). DMSO (1%) also induces  
*mda-6* by 3 h and augmented expression persists at day 5  
when cells are terminally differentiated (data not  
35     shown). These results indicate that induction of both

-268-

macrophage/monocyte and granulocyte differentiation pathways in human myeloid leukemia HL-60 cells results in the induction of *mda-6* expression. In addition, *mda-6* expression is induced during the early commitment stage of HL-60 differentiation and persists in terminally differentiated cells.

To determine if the elevation in *mda-6* expression with an increase in MDA-6 (WAF1/CIP1/SDI1) protein, HL-60 cells were labeled for 4 h with <sup>35</sup>S-methionine after 12, 24, 48 and 72 h treatment with TPA (3 nM), DMSO (1%) or RA (1 μM) and lysates were immunoprecipitated using WAF1/CIP1 antibody (Fig. 28). As a control for protein loading, the level of ACTIN protein was determined by immunoprecipitation. Although no MDA-6 protein was detected in HL-60 cells, 12 h treatment with TPA or RA resulted in immunologically reactive MDA-6 protein. In 1% DMSO treated HL-60, MDA-6 protein was first apparent by 48 h. The levels of MDA-6 protein increased in a temporal manner with all three inducers and the highest levels were apparent at 72 h. The most active inducer of MDA-6 protein, as well as the most active growth suppressing agent, was TPA. Polyclonal antibodies prepared against N-terminal peptide regions of MDA-6 also immunoprecipitated MDA-6 from differentiation inducer treated HL-60 cells and human melanoma cells. In contrast, using a monoclonal antibody (PAb 421) that reacts with both wild-type and mutant p53, no reactive protein was detected after immunoprecipitation of <sup>35</sup>S-methionine labeled lysates prepared from HL-60 cells and TPA-, DMSO- or RA-treated HL-60 cells (data not shown). These results provide direct evidence that induction of elevated *mda-6* mRNA expression in differentiation inducer-treated HL-60 cells results in elevated MDA-6

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-269-

protein levels in the absence of p53 protein.

Induction of *mda-6* expression is altered in differentiation-resistant HL-60 variants

5 The availability of variants of HL-60 cells displaying resistance to TPA-induced growth suppression and differentiation (Murao et al., 1983; Fisher et al., 1984; Anderson et al., 1985; Mitchell et al., 1986; Homma et al., 1986, 1988; Tonetti et al., 1992) provides a  
10 valuable experimental model to evaluate the potential involvement of *mda-6* in these processes. By continuously growing HL-60 cells in gradually increasing concentrations of TPA (up to 3  $\mu$ M), the TPA-resistant HL-  
15 60 variant HL-525 was developed (Homma et al., 1986; Mitchell et al., 1986). These cells were used to determine the kinetics of *mda-6* expression as a function of short term (1 through 12 h) and long-term (1 through 6 d) treatment with TPA, Vit D3 and RA. As anticipated,  
20 HL-525 cells demonstrate a suppressed response to *mda-6* induction following TPA treatment (Fig. 26A-C). Parental HL-60 cells treated with 3 nM TPA show *mda-6* expression within 2 h, whereas induction in HL-525 cells is not apparent until 12 h treatment (Figs. 26A-C and 27A-C).  
25 Vit D3 (400 nM) treatment of HL-60 parental cells results in *mda-6* expression after 1 h with a continued increase over the 12 h test period (Fig. 26A-C). In HL-525 cells, *mda-6* expression is observed by 2 h following Vit D3 treatment and the level of expression in the variant  
30 cells after 12 h exposure to 400 nM Vit D3 is lower than seen in HL-60 cells similarly treated for 1 h. RA (1  $\mu$ M) induces *mda-6* expression after 3 h in HL-60 cells and the level of *mda-6* expression increases over the 12 h test period. In contrast, no induction of *mda-6* is apparent  
35 in RA-treated HL-525 cells by 12 h. These results

-270-

indicate that the TPA-resistant HL-525 cells differ from HL-60 cells with respect to the early induction of *mda-6*. This defective early induction of *mda-6* in HL-525 cells is most evident following RA or TPA treatment, whereas  
5 Vit D3 results in a reduced capacity to induce *mda-6* that is less dramatic than with the other inducers. Since these studies involve separate RT-PCR reactions, direct quantitation is difficult, however, it appears that both  
10 the early kinetics of induction and the final level of induction of *mda-6* are reduced in HL-525 cells in comparison with HL-60 parental cells. These observations are supported by Northern blotting analyses of similar samples (data not shown).

15 RT-PCR analyses were used to determine the effect of extended treatment times (12 h to 6 d) on induction of *mda-6* in HL-60 and HL-525 cells (Fig. 27A-C). With the three inducers, *mda-6* expression in HL-60 cells was apparent during all of the extended treatment times. In  
20 the case of HL-525, *mda-6* expression was also induced at all of the time points by TPA and Vit D3. On the basis of direct quantitation and an adjustment for amplification of GAPDH, the levels of *mda-6* induction in TPA- and Vit D3-treated HL-525 cells appear to be lower  
25 than in similarly treated HL-60 cells (Fig. 27A-C). However, since RT-PCR assay employed is not quantitative (no internal GAPDH control was used in the same amplification reaction), further studies using quantitative RT-PCR and Northern blotting will be  
30 required to verify this conclusion. In the case of RA treated HL-525 variant cells, a delay in induction of *mda-6* is evident, i.e. expression is not detected until 2 d post-treatment (Fig. 27A-C). These results suggest that the HL-525 TPA-resistant variant may also display an  
35 attenuated response to *mda-6* expression following

-271-

extended treatments with RA and Vit D3 as well as TPA.

Previous studies indicate that a 6 d growth of HL-60 cells is strongly inhibited (60 to 98% reduction in cell number relative to control) by 3 nM TPA, 1  $\mu$ M RA and 400 nM Vit D3 with TPA being the most effective of the group (Murao et al., 1983 and data not shown). In the HL-525 variant, only a slight reduction (less than 5%) in growth is observed after treatment with TPA or Vit D3. Treatment with 1  $\mu$ M RA for 6 d results in a 35% reduction in cell numbers. These results indicate that the TPA-resistant variant displays some cross-resistance to the growth suppressive effects of RA and it is relatively refractive to the dose of Vit D3 used for the present studies. Analysis of OKM1 reactivity, which reacts with human blood monocytes and granulocytes (Foon et al., 1982), in HL-60 indicates that both TPA and Vit D3 are similarly active, while RA displays a somewhat reduced activity (Table 4). In HL-60 cells, an increase in OKM1 positive cells is seen with all of the inducers over time, with maximum induction observed at 6 d with TPA and Vit D3 and by 4 d with RA. In contrast, HL-525 cells treated with TPA do not display a significant increase in OKM1 positive cells, whereas RA induces a small effect at 6 d and Vit D3 is an effective inducer of OKM1 reactivity at both 4 and 6 d. These observations indicate that the effects of Vit D3 on growth suppression and induction of differentiation are not directly correlated processes in HL-525 cells, whereas growth suppression and induction of differentiation are related changes in Vit D3 treated HL-60 parental cells.

-272-

Table 4

OKM1 reactivity in HL-60 and HL-525 cells treated with differentiation inducers

5	Cell type	Inducer	Days of induction		
			2	4	6
10	HL-60	Untreated	3	5	12
		TPA	55	81	95
		RA	49	76	77
		Vit D3	-	88	95
15	HL-525	Untreated	5	3	5
		TPA	-	8	4
		RA	-	10	13
		Vit D3	-	84	80

20. Cells were seeded into 100-mm Petri dishes at  $1.5 \times 10^5$  cells/ml and TPA (3 nM), RA (1  $\mu$ M) or Vit D3 (400 nM) added 12 h later. OKM1 reactivity was assessed at the indicated times as described in Materials and methods.

25. Results are expressed as percent OKM1 positive cells.

- = Not determined.

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-273-

mda-6 is an immediate-early gene induced in HL-60 cells in the presence of cycloheximide.

5 To determine if the induction of *mda-6* expression requires continuous protein synthesis, the effect of the protein synthesis inhibitor cycloheximide (CHX) on de novo and TPA-induced *mda-6* expression was determined (Fig. 29A-B). Treatment of HL-60 cells with CHX for 1,  
10 3, 6 or 10 h results in the induction of *mda-6*. In addition, the relative level of *mda-6* induction is unaffected when HL-60 cells are simultaneously treated with TPA and CHX. In HL-525 cells, CHX also induces *mda-6* expression, but the level of induction is lower than in  
15 HL-60 parental cells. In contrast, when HL-525 cells are treated with both CHX and TPA, *mda-6* expression is increase (superinduction) to a greater extent than with CHX alone (Fig. 29A-B). These results indicated that *mda-6* is an immediate-early response gene in HL-60 cells and induction does not require ongoing protein synthesis. The ability of CHX alone to induce *mda-6* expression in HL-60 and to a lesser extent in HL-525 cells suggests that *mda-6* expression may be controlled by an unstable suppressor. In the case of HL-525, inhibition in TPA-  
20 induced differentiation may be related to alterations in the levels of this unstable suppressor.  
25

#### Experimental Discussion

30 Terminal differentiation in diverse cell types occurring either spontaneously or as a consequence of treatment with specific inducing agents correlates with an irreversible loss of proliferative potential (Sachs, 1978; Jimenez & Yunis, 1987; Waxman et al., 1988; 1991;  
35 Fisher & Rowley, 1991; Hoffman & Liebermann, 1994). The

-274-

specific gene expression changes and proteins that mediate growth arrest and induction of differentiation in the majority of differentiation models remain to be defined. Using subtraction hybridization and agents capable of inducing terminal cell differentiation in human melanoma cells, *mda* genes have been identified whose expression directly correlate with growth arrest and terminal cell differentiation (Jiang & Fisher, 1993; Jiang et al., 1994). One such gene, *mda-6* is identical to WAF1/CIP1/CAP20/SDI1 that encodes the ubiquitous inhibitor of cyclin-dependent kinases, p21 (Jiang & Fisher, 1993; Jiang et al., 1994). A direct effect of p21 on growth has been demonstrated by transfecting expression constructs into mammalian cells (El-Deiry et al., 1993; Harper et al., 1993; Jiang et al., in preparation). Although induction of p21 expression was initially considered to be dependent upon wild-type p53 protein (El-Deiry et al., 1993, 1994), recent studies suggest that this assumption must be reevaluated (Michieli et al., 1994). These include the ability of mitogens to transiently stimulate p21 expression in quiescent fibroblasts from p53 knock out mice lacking p53 protein (Michieli et al., 1994) and the decreased expression of p53 mRNA and protein but the increased expression of *mda-6* mRNA and protein in terminally differentiated human melanoma cells (Jiang et al., in preparation). In the present study, definitive evidence is presented that p21 (*mda-6*/WAF1/CIP1/CAP20/SDI1) is an immediate-early response gene that is induced in the absence of p53 protein as a function of growth arrest and induction of differentiation in HL-60 cells. The ability of diverse inducers, including TPA and Vit D3 that produce monocyte and macrophage differentiation and RA and DMSO that elicit granulocyte differentiation, to stimulate p21 mRNA synthesis and p21 protein in HL-60

-275-

cells defines this early genotypic change as an important component of growth arrest and terminal differentiation in myeloid leukemic cells.

5 Previous studies have demonstrated that TPA-resistant variants of HL-60 cells, isolated in the absence of mutagenesis, display a number of biochemical and cellular traits that distinguish them from parental TPA-sensitive  
10 HL-60 cells (Murao et al., 1983; Fisher et al., 1984; Anderson et al., 1985; Homma et al., 1988; Tonetti et al., 1992). TPA-resistant HL-60 variant cells, such as HL-525, display altered protein kinase C (PKC) isozyme profiles, including the absence of PKC- $\beta$  and possibly a  
15  $\delta$ -like PKC gene expression (Tonetti et al., 1992). TPA resistance in HL-60 cells is associated with decreased fluidity of either the inner leaflet of the plasma membrane and/or of the cytosolic organellar membranes (Fisher et al., 1984). A striking biochemical difference  
20 between HL-60 and TPA-resistant HL-60 variants is the inability of the latter cell type to translocate PKC from the cytosol to the membrane fraction following TPA treatment (Homma et al., 1986). TPA-resistant HL-60 variants also display modifications in protein phosphorylation patterns after TPA treatment (Homma et  
25 al., 1988) and altered responses in immediate-early gene expression following TPA treatment (Tonetti et al., 1992). Of most direct relevance to the present study is the observation by Tonetti et al. (1992) that the HL-525 variant displays an attenuated response to TPA induction  
30 of immediate-early genes, including *c-fos*, *c-jun* and *jun-B*. The level of *c-fos* and *jun-B* induction is substantially greater in TPA-treated HL-60 parental cells and in the TPA-sensitive HL-60 clone, HL-205, than in two TPA-resistant clones, HL-525 and HL-534. In the case of  
35 *c-jun*, TPA fails to induce this gene expression change in

-276-

HL-525 or HL-534 cells, whereas induction is apparent in HL-60 and HL-205 cells by 9 h post-treatment with TPA (Tonetti et al., 1992).

5 In the present study, applicants demonstrate that the TPA-resistant variant HL-525 displays a diminished response to TPA-induction of *mda-6* in comparison with HL-60 cells (Figs. 26A-C and 27A-C). The HL-525 cells likewise differ from HL-60 cells in the induction of *mda-*  
10 6 following treatment with Vit D3 and RA (Figs. 26A-C and 27A-C). With both of these agents, the temporal pattern of induction and the magnitude of induction of *mda-6* are diminished in HL-525 cells. The TPA-resistant HL-525 variant also displays a reduced susceptibility to growth  
15 arrest following treatment with Vit D3 and RA, whereas Vit D3 still has the capacity to induce differentiation as monitored by OKT1 reactivity (Table 4). These findings indicate that these two phenomena, i.e., growth arrest and differentiation, are dissociable processes in  
20 HL-525 cells. Unlike TPA- and RA-treated HL-525 cells, Vit D3 treated cells show early induction of *mda-6* (after 2 h treatment) (Fig. 26A-C). Although further studies are required, it is tempting to speculate that the early induction of *mda-6* by Vit D3 may be a primary determinant  
25 committing HL-525 cells to differentiate, whereas the absence of sufficient accumulated levels of p21 protein, encoded by *mda-6*, in long-term treated cultures (6 d) precludes growth arrest.

30 CHX induces *mda-6* in HL-60 cells and the ability of TPA to induce *mda-6* expression is not inhibited by CHX indicating that *mda-6* is an immediate-early response gene. In HL-525 cells, CHX induces *mda-6* less effectively than in HL-60 cells, whereas the combination  
35 of CHX and TPA results in a superinduction of *mda-6* in

-277-

this TPA-resistant variant (Fig. 29A-B). These observations and previous investigations support the hypothesis that the HL-525 variant cells may be defective in signal transduction processes, possibly involving PKC- $\beta$  and a  $\delta$ -like PKC gene, that prevent or reduce the induction of immediate-early response genes, including *c-fos*, *c-jun*, *jun-B* and *p21*. The lower levels of the immediate-early response genes following TPA treatment may then impede the induction of subsequent cellular genes involved in the initiation of terminal differentiation in HL-525 cells. Induction of the immediate-early gene *p21* during the commitment phase of HL-60 differentiation may be an important component in initiating differentiation, whereas a continued elevation of *p21* may be required for growth arrest and maintenance of terminal differentiation in HL-60 cells. In the case of the HL-525 variant, treatment with TPA does not induce the early induction of *p21* and it may not generate sufficiently high levels of *p21* to produce sustained growth arrest.

Recent studies are providing new insights into the mode of action of *p21*. The *p21* protein was originally identified as part of quaternary cyclin D complexes in human diploid fibroblasts, that also possess cyclin-dependent kinases (CDK) and proliferating cell nuclear antigen (PCNA) (Xiong et al., 1992). Subsequent studies demonstrated that *p21* and PCNA can form multiple quaternary complexes with all cyclins and CDKs in normal human fibroblasts, but not in virally transformed cells (Xiong et al., 1993a). *p21* has also been shown to associate with and inhibit the activity of all cyclin-CDK enzymes (Xiong et al., 1993b; Harper et al., 1993; Gu et al., 1993). Recent experiments demonstrate that *p21* can directly complex with and inhibit PCNA suggesting that

-278-

this protein may be a critical regulator of DNA replication, DNA repair and cell cycle machinery (Waga et al., 1994). The importance of p21 in cell cycle and growth control has been reinforced by the independent isolation of this gene by virtue of its induction by the tumor suppressor p53 [WAF1, (El-Deiry et al., 1993)], as a direct regulator of CDK2 using the two-hybrid screening technique [CIP1, Harper et al., 1993], as a cDNA from senescent cells with the ability to inhibit the ability of young cells to enter S phase after overexpression following transient transfection [SDI1, (Noda et al., 1994)] and as a differentially expressed differentiation related cDNA isolated from a human melanoma cell library using subtraction hybridization (mda-6, Jiang & Fisher, 1993; Jiang et al., 1994). The level of p21 has been shown to vary depending on the specific stage of the cell cycle (Li et al., 1994). In IMR90 normal diploid fibroblast cells released from serum starvation, the levels of p21 are maximum immediately after serum stimulation, start to decrease as cells reach the G1/S boundary, display lowest levels during S phase, and increase again as cells leave the S phase and enter the G2 and M phase (Li et al., 1994). These observations indicate that p21 may contribute to both the G1/S and the G2/M checkpoint pathways. The interaction of p21 with cyclin and CDK during the cell cycle is not random, but rather occurs when the specific cyclin-CDK enzyme is reputed to function (Li et al., 1994). Moreover, the increased level of p21 in quiescent and terminally differentiated cells suggests that this protein may play a crucial role in preventing these cells from re-entering the cell cycle, an absolute requirement for terminal differentiation.

In summary, the ability of different inducers of growth

-279-

suppression and terminal differentiation to induce p21 early in the differentiation process and the persistence of elevated levels of p21 after terminal differentiation in the p53 negative HL-60 cell line indicates an important role for this inhibitor of cyclin-dependent kinases in differentiation. Further support for an involvement of p21 in growth control and differentiation is indicated by the ability of structurally diverse inducers of differentiation to induce p21 expression and growth arrest in additional cell lines, including human melanomas (Jiang & Fisher, 1993; Jiang et al., 1994) and human neuroblastomas (Jiang et al., in preparation). Moreover, the diminished ability of specific inducers of differentiation to produce growth arrest and differentiation in the TPA-resistant HL-525 variant also correlates with a reduced early and sustained induction of p21. Further studies to determine if forced expression of p21, using inducible expression vectors, are sufficient to induce an irreversible loss in proliferative capacity and terminal differentiation in HL-60 cells, and other differentiation competent cell culture systems, appears warranted and are currently in progress. These experiments will permit a direct functional evaluation of p21 in regulating both cellular growth and differentiation in the absence and presence of wild-type p53.

#### Materials and Methods

##### Cells and culture conditions

HL-60 cells were originally provided by Dr. R. C. Gallo (National Cancer Institute, Bethesda, MD) (Collins et al., 1978; Huberman & Callahan, 1979). HL-60 cells designated HL-525 were derived by cloning HL-60 cells

-280-

after subculturing 102 times in the presence of increasing concentrations (up to 3  $\mu$ M) of TPA at 5- to 8-day intervals (Homma et al., 1986; Mitchell et al., 1986). The HL-525 cell variant displays a stable phenotype with regards to resistance to induction of cell differentiation by TPA for at least 50 to 60 subcultures (200 to 300 cell generations). Prior to the experiments described in this study, the HL-525 cells were subcultured more than 20 times in the absence of TPA.

Cells were grown in 100-mm tissue culture dishes in RPMI 1640 medium supplemented with 20% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml) (Grand Island Biological Co., NY) at 37°C in an atmosphere of 5% CO<sub>2</sub> in air in a humidified incubator.

The 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Vit D3) and TPA were dissolved in a final concentration of 0.01% DMSO, and all-trans retinoic acid (RA) was dissolved in 0.1% DMSO in culture medium. DMSO at these concentrations did not affect cell growth or the expression of the various differentiation markers.

Control cultures were treated with DMSO at a final concentration of 0.1% in culture medium. For experiments designed to test the effect of DMSO on HL-60 and HL-525 cells, DMSO was added to tissue culture medium at a final concentration of 1%. Stock solutions of CHX (10 mg/ml) were prepared in culture medium. CHX was added at a final concentration of 10  $\mu$ g/ml. To examine a requirement for protein synthesis on *mda-6* gene expression, HL-60 or HL-525 cells were seeded into 150 mm Petri dishes (5 X 10<sup>5</sup> cells/ml) in 30 ml of medium. CHX was added to a final concentration of 10  $\mu$ g/ml 15 min prior to the addition of TPA to 3 nM. Cells were harvested at various times after addition of TPA for subsequent RNA isolation and analysis.



-281-

Measurement of differentiation and growth

Cell counts were determined by hemocytometer chamber counting. Immunofluorescence tests for reactivity with the OKM1 antibody (Ortho Pharmaceutical Corp., Raritan, NJ) were performed as previously described (Murao et al., 1983).

RNA isolation, Northern blotting and RT-PCR

RNA was purified by centrifugation through a CsCl cushion as described by Chirgwin et al. (1979). Ten  $\mu$ g of RNA was denatured with glyoxal/DMSO, electrophoresed on 1.0% agarose gels, transferred to nylon membranes, hybridized to a  $^{32}$ P-labeled *mda-6* probe (Jiang and Fisher, 1993) and then after stripping the membrane hybridized to a  $^{32}$ P-labeled rat GAPDH probe (Fort et al., 1985), as described previously (Reddy et al., 1991; Su et al., 1991; Jiang et al., 1992). Following hybridization, the filters were washed and exposed for autoradiography (Reddy et al., 1991; Su et al., 1991; Jiang et al., 1992). *mda-6* and GAPDH gene expression were also determined by reverse transcription-polymerase chain reaction (RT-PCR) as described (Adollahi et al. 1991; Lin et al., 1994). Total cytoplasmic RNA was treated with 0.5 units DNase (Boehringer-Mannheim Biochemicals)/ $\mu$ g RNA in 15% glycerol, 10 mM Tris, pH 7.5, 2.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 80 mM KCl, 1 mM CaCl<sub>2</sub>, and 1 unit/ml RNasin (Promega) at 30°C for 10 min. RNA was extracted with phenol-chloroform, precipitated with sodium acetate/ethanol and RNA pellets were resuspended in diethylpyrocarbonate-treated H<sub>2</sub>O. One  $\mu$ g of total RNA was reverse transcribed with 200 units of murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) in 20  $\mu$ l

-282-

containing 1 mM deoxyribonucleotide triphosphates, 4 mM MgCl<sub>2</sub>, 10 mM Tris, pH 8.3, 50 mM KCl, 0.001% gelatin and 0.2 µg oligo-dT primer. Samples were diluted to 100 µl with buffer containing 0.2 mM deoxyribonucleotide triphosphates, 2 mM MgCl<sub>2</sub>, 10 mM Tris, pH 8.3, 50 mM KCl and 0.001% gelatin. Fifty pmol of each primer, 1.5 units Taq DNA polymerase (Perkin-Elmer Cetus) were added and samples were covered with mineral oil, heated at 95°C for 5 min and subjected to 20 cycles of PCR in a Perkin-Elmer Thermal Cycler using 2 min denaturation at 95°C, 1 min annealing at 55°C and 4 min polymerization at 72°C. After extraction with chloroform, 20 µg of products were electrophoresed, blotted onto nylon filters and hybridized with an *mda-6* or GAPDH specific probe. The template primers for *mda-6* were 5' to 3' CTCCAAGTACACTAAGCACT and TAGTTCTACCTCAGGCAGCT (GenBank accession number U09579) and the template primers for human GAPDH were 5' to 3' CATGGCCTCCAAGGAGTAAGA and CGTCTTCACCACCATGGAGAA (GenBank accession number J02642).

#### Immunoprecipitation Analyses

Immunoprecipitation analyses were performed as described previously (Duigou et al., 1991; Su et al. 1993). Logarithmically growing HL-60 cells were either untreated or treated for 12, 24, 48 or 72 h with TPA (3 mM), RA (1 µM) or DMSO (1%) in 10-cm plates. Cultures were starved of methionine for 1 h at 37°C in methionine-free medium, cells were concentrated by pelleting and labeled for 4 h at 37°C in 1 ml of the same medium with 100 µCi of [<sup>35</sup>S] (NEN; Express <sup>35</sup>S). After labeling, the cells were washed twice with ice-cold phosphate-buffered saline and lysed for 1 h on ice by the addition of RIPC (20 mM Tris-base, pH 7.5, 500 mM NaCl, 0.05% Nonidet P-40, 100 µg/ml

-283-

phenylmethanesulfonyl fluoride and 0.02% sodium azide). The lysate was clarified by centrifugation in an Eppendorf microfuge at 10,000 X g for 10 min at 4°C. Samples containing  $4 \times 10^6$  counts were incubated with 2  $\mu$ g of WAF1/CIP1 (C-19) (Santa Cruz Biotechnology) (or MDA-6 peptide-derived) rabbit polyclonal IgG or actin monoclonal antibody (Oncogene Sciences) with rocking at 4°C for 24 h. The next day, 30  $\mu$ l (packed volume) of protein G-agarose (Oncogene Sciences) was added to each tube, and incubation with rocking at 4°C continued for another hour. The protein G pellets were then washed five times with 1 ml of ice-cold RIPC:phosphate-buffered saline (1:1, v/v). Thirty  $\mu$ l of sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer were added to the pellets, and the sample was heated at 87°C for 3 min. The samples were loaded onto an 10% polyacrylamide gel and run overnight at 40 V. The gel contained Rainbow protein markers (Amersham) for sizing. Gels were fixed with 10% acetic acid plus 10% methanol for 30 min, incubated in DMSO for 30 min, incubated with 10% 2,5-diphenyloxazole in DMSO for 30 min, washed three times with cold water (10 min each), dried and exposed to film.

-284-

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-285-

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-286-

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-287-

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-288-

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-289-

Seventh Series of Experiments

The combination of recombinant human fibroblast interferon (IFN- $\beta$ ) and the antileukemic compound mezerein (MEZ) induces terminal differentiation with an irreversible loss of proliferative capacity in human melanoma cells. Using subtraction hybridization, cDNAs were identified that display enhanced expression in terminally differentiated and growth arrested human melanoma cells (Jiang and Fisher, 1993; Jiang et al., 1994). A specific melanoma differentiation-associated (*mda*) cDNA, *mda-6*, is described whose expression inversely correlates with melanoma progression and growth. *mda-6* is identical to WAF1/CIP1/SDI1 that encodes the M<sub>r</sub>21,000 protein (p21) that is an inhibitor of cyclin-dependent kinases. Actively growing normal melanocyte, SV40-immortalized human melanocyte and dysplastic nevus cell lines synthesize elevated levels of *mda-6* mRNA; whereas, actively proliferating radial and early vertical growth phase primary melanomas as well as metastatic human melanoma cells produce reduced levels of *mda-6* mRNA. Treatment of primary and metastatic human melanoma cells with IFN- $\beta$  + MEZ results in growth inhibition and an increase in *mda-6* expression. *mda-6* expression also increases when human melanoma cells are grown to high saturation densities or when grown in serum-free medium. Using anti-p53 and anti-p21 antibodies, an inverse correlation is found between p53 and p21 protein levels during growth arrest and differentiation. Induction of growth arrest and terminal differentiation in H0-1 human melanoma cells by IFN- + MEZ results in a temporal decrease in wild-type p53 protein levels with a corresponding increase in p21 levels. In the Matrigel-assisted melanoma progression

-290-

model, *mda-6* expression decreases in early vertical growth phase primary human melanoma cells selected for autonomous or enhanced tumor formation in nude mice. In metastatic human melanoma cells displaying a loss of  
5 metastatic potential resulting following introduction of a normal human chromosome 6, *mda-6* mRNA levels increase. Taken together, these studies indicate that *mda-6* (p21) may function as a negative regulator of melanoma growth, progression and metastasis.

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Development of malignant melanoma in humans, with the exception of nodular type melanoma, often consists of a series of sequential alterations in the evolving tumor cells (for reviews see: Kerbel, 1990; Herlyn, 1990;  
15 Clark, 1991). These changes may include conversion of a normal melanocyte into a common acquired melanocytic nevus (mole), followed by the development of a dysplastic nevus, a radial growth phase (RGP) primary melanoma, a vertical growth phase (VGP) melanoma and ultimately a  
20 metastatic melanoma. Melanomas are readily treatable during the early stages of development; however currently employed therapies are not very effective (<20% survival) in preventing metastatic spread and morbidity in patients with VGP lesions >4.0-mm thickness. These observations  
25 indicate an imperative for improved therapeutic modalities for treating patients with this malignancy.

A potentially less toxic strategy for cancer therapy involves a procedure termed "differentiation therapy"  
30 (Sachs, 1978; Jimenez & Yunis, 1987; Waxman et al., 1988, 1991; Fisher & Rowley, 1991; Lotan, 1993; Jiang et al., 1994a). An essential premise underlying this approach is that specific cancers have reversible defects in normal programs of differentiation and growth control. By using  
35 specific single or combinations of agents, it has been

-291-

possible to slow or stop proliferation of cancer cells and correspondingly increase expression of differentiation-associated properties (for reviews see: Waxman et al., 1988, 1991; Fisher & Rowley, 1991; Lotan, 1993; Jiang et al., 1994a).

Treatment of human melanoma cells with IFN- $\beta$  + MEZ results in rapid, irreversible loss of proliferative capacity, an induction of specific changes in gene expression, a modification in cell surface antigenic profile and terminal cell differentiation (Fisher et al., 1985, 1986; Guarini et al., 1989, 1992; Graham et al., 1991; Jiang & Fisher, 1993; Jiang et al., 1993, 1994a). The ability of IFN- $\beta$  + MEZ to induce terminal differentiation in human melanoma cells is hypothesized to be a consequence of activation by these agents of genes that negatively control melanoma growth and induce differentiation. Two predictions arise from this model: specific genes involved in growth control and differentiation in human melanoma cells are expressed at diminished levels in melanoma versus their normal melanocyte counterpart; and the combination of IFN- $\beta$  + MEZ can induce enhanced expression of specific melanoma differentiation associated (*mda*) genes, growth suppression and terminal cell differentiation (Jiang & Fisher, 1993; Jiang et al., 1994a). To test these possibilities and to directly identify and clone genes that would fulfill this scheme, a modified subtraction hybridization approach was used that employed the human melanoma cell line H0-1 treated with IFN- $\beta$  + MEZ (Jiang & Fisher, 1993). Using this strategy a series of melanoma differentiation associated (*mda*) genes were cloned that display enhanced expression in H0-1 human melanoma cells treated with IFN- $\beta$  + MEZ (Jiang & Fisher, 1993; Jiang et al., 1994a).

-292-

*mda-6* encodes the cyclin dependent kinase inhibitor protein, p21 (Jiang et al., 1994a, 1994b). p21 has been cloned by a number of laboratories by virtue of its ability to interact with and inhibit cyclin dependent kinases (CIP1: cyclin-dependent kinase (CDK)-interacting protein-1) (Harper et al., 1993), its induction by wild-type p53 (WAF1; wild-type (wt) p53 activated factor-1) (El-Deiry et al., 1993), its induction during senescence (SDI1; senescent cell-derived inhibitor-1) (Noda et al., 1994) and its induction as a function of growth arrest and terminal differentiation in human melanoma cells (*mda-6*; melanoma differentiation associated gene-6) (Jiang & Fisher, 1993; Jiang et al., 1994a). Although originally assumed to be dependent on wt p53 for induction (El-Deiry et al., 1993, 1994), recent studies indicate that p21 can also be induced in a p53-independent manner (Michieli et al., 1994; Jiang et al., 1994b; Steinman et al., 1994). In the present study, applicants demonstrate that the levels of p21 protein increase as the levels of wt p53 protein decrease during IFN- $\beta$  + MEZ induction of growth suppression and terminal differentiation in H0-1 human melanoma cells. These results reveal a novel association between p21 and p53 in the process of melanoma growth and differentiation. Moreover, applicants show a relationship between expression of *mda-6* and melanoma evolution, differentiation and growth. These results provide a direct link between alterations in p21 expression and cancer progression.

### Experimental Results

#### Increased *mda-6* expression in human melanoma cells during growth inhibition and terminal cell differentiation

-293-

To define the molecular basis by which IFN- $\beta$  + MEZ induces irreversible growth arrest and terminal differentiation in human melanoma cells applicants used the technique of subtraction hybridization with uninduced and differentiation inducer treated H0-1 human melanoma cDNA libraries (Jiang & Fisher, 1993). Using this strategy, an *mda-6* cDNA was identified in a differentiation inducer (IFN- $\beta$  + MEZ) treated subtracted H0-1 human melanoma library that displays differential expression as a function of IFN- $\beta$  + MEZ induced growth arrest and terminal differentiation (Jiang & Fisher, 1993; Jiang et al., 1994a). By screening a differentiation inducer-treated H0-1 cDNA library (Jiang & Fisher, 1993) and using rapid amplification of cDNA ends (RACE) (Frohman et al., 1988; Loh et al., 1989; Ohara et al., 1989) a full-length *mda-6* cDNA was cloned (Jiang et al., 1994a). *mda-6* contains the same open reading frame as WAF1 (El-Deiry et al., 1993), CIP1 (Harper et al., 1993), CAP20 (Gu et al., 1993) and SDI1 (Noda et al., 1994) (Fig. 30). These genes encode the ubiquitous inhibitor of cyclin dependent kinases, p21.

Treatment of actively growing H0-1 cells for 24 h with IFN- $\beta$  + MEZ results in increased *mda-6* mRNA levels and growth inhibition (Fisher et al., 1985; Jiang & Fisher, 1993; Jiang et al., 1993) (Fig. 31A-E). In contrast, treatment of H0-1 cells with either IFN- $\beta$  or MEZ for 24 h results in a smaller induction of *mda-6* expression and reduced growth inhibition (Fisher et al., 1985; Jiang & Fisher, 1993; Jiang et al., 1993). Enhanced *mda-6* expression and terminal cell differentiation are also produced in additional human melanoma cells treated with IFN- $\beta$  + MEZ, including F0-1, SH-1, L0-1, WM-239 and WM-278 (Fig. 32). *mda-6* is expressed at higher de novo

-294-

levels in a low-density SV40-transformed human melanocyte culture (FM516-SV) (Melber et al., 1989) than in low-density and the majority of high-density melanoma cultures (Fig. 32 and data not shown). *mda-6* levels are increased and melanoma growth is inhibited after 24 h treatment with IFN- $\beta$  + MEZ and 96 h treatment with the inducers results in terminal differentiation in the human melanoma cultures (Fig. 32 and data not shown). In contrast, although *mda-6* expression is enhanced by 24 h in IFN- $\beta$  + MEZ-treated FM516-SV cells, growth is only marginally reduced ( $\leq 15\%$ ) (Fig. 32 and data not shown). Unlike human melanoma cells, 96 h exposure of FM516-SV cells to IFN- $\beta$  + MEZ does not result in terminal differentiation in the majority of treated cells (data not shown).

Elevated *mda-6* expression persists in H0-1 cells, and other melanoma cells, induced to terminally differentiate by continuous exposure to IFN- $\beta$  + MEZ for 96 h, whereas H0-1 cells, and additional melanoma cultures, treated singly with IFN- $\beta$  or MEZ for 96 h recover from growth suppression and contain similar levels of *mda-6* as control cells (Fig. 31A-E and data not shown). Previous studies indicate that growth of H0-1 cells for 24 h in IFN- $\beta$  + MEZ or MEZ, but not IFN- $\beta$ , followed by removal of the inducing agent(s) and growth for an additional 72 h in complete medium results in sustained growth inhibition (Jiang et al., 1993). Under these experimental conditions, *mda-6* expression remains elevated with both types of treatment with the highest expression occurring with IFN- $\beta$  + MEZ that also induces the greatest residual growth inhibition (Jiang et al., 1993) (Fig. 31A-E).

Even without treatment with differentiation-inducing agents, H0-1 cells grown to high density (Fig. 31A-E) or

-295-

grown in the absence of serum (Fig. 31A-E) express elevated levels of *mda-6* mRNA. The induction of enhanced *mda-6* mRNA levels is rapid, occurring within 15 min treatment (Fig. 31A-E). Elevated *mda-6* expression is also  
5 evident in IFN- $\beta$  + MEZ treated H0-1 cells simultaneously cultured in the presence of cycloheximide (data not shown) and following a 2 h exposure to 25  $\mu$ g/ml of the alkylating carcinogen, methyl methanesulfonate (data not shown). These observations suggest a direct relationship  
10 between *mda-6* expression and growth suppression in human melanoma cells.

Inverse relationship between p21 and wild-type p53 levels during growth arrest and differentiation in H0-1 human melanoma cells  
15

Treatment of H0-1 cells with IFN- $\beta$  + MEZ results in rapid growth arrest, that is apparent within 24 h (Fisher et al., 1985; Jiang et al., 1993). In contrast, IFN- $\beta$  and  
20 MEZ alone result in smaller changes in growth in H0-1 cells (Fisher et al., 1985; Jiang et al., 1993). Analysis of p53 mRNA levels in IFN- $\beta$ , MEZ and IFN- $\beta$  + MEZ treated H0-1 cells indicate no significant change after 24 h, but significant inhibition is observed in p53 expression that  
25 is maximum in IFN- $\beta$  + MEZ (96 h) treated terminally differentiated H0-1 cells (Jiang et al., in preparation). MEZ also induces a reduction in p53 mRNA levels in 96 h treated H0-1 cells, whereas no change in p53 mRNA occurs in H0-1 cells treated with IFN- $\beta$  for 96 h (Jiang et al.,  
30 in preparation). Since the kinetics of suppression in p53 gene expression is the opposite of that observed with *mda-6* expression in differentiation inducer treated H0-1 cells, experiments were conducted to determine the effects of growth suppression and terminal  
35 differentiation on p53 and p21 protein levels.

-296-

Immunoprecipitation analysis of p53 under conditions preventing protein denaturization (< 1% SDS) with monoclonal antibodies Ab1 (PAb421; Oncogene Sciences), that identifies both wild-type and mutant p53, and Ab3 (PAb240; Oncogene Sciences) that recognizes mutant p53, indicate that H0-1 cells contain a wild-type p53 protein (data not shown). A wild-type p53 protein is also present in a number of other cell types evaluated in the present study, including FM516-SV, L0-1, SH-1 and F0-1, whereas WM239 cells contain a mutant p53 (data not shown). To rule out potential artifacts, immunoprecipitation studies with Ab1 and Ab3 were performed with labeled extracts from cell lines with known p53 status, including MeWo (previously shown to contain a mutant p53 by sequence analysis) (Loganzo et al., 1994), Saos-2 (p53-null phenotype), human skin fibroblasts (wild-type p53) and SW480 colon carcinoma cells (mutant p53) (data not shown). The current results are in agreement with several recent studies (Volkenandt et al., 1991; Castresana et al., 1993; Greenblatt et al., 1994; Montano et al., 1994; Loganzo et al., 1994) indicating that p53 mutations are rare in human melanoma and the majority of human melanomas contain a wild-type as opposed to a mutant p53 protein.

To determine the effect of the various inducing agents on p53 and p21 protein levels the following experiment was performed. H0-1 cells were grown in inducer-free medium (control), IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml) or IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml) for 24, 48, 72 or 96 h, cells were labeled with  $^{35}\text{S}$ -methionine and cell lysates were prepared and analyzed by immunoprecipitation analyses using Ab1 (PAb421), p21 (WAF1/CIP1, Santa Cruz Biotechnology; and rabbit polyclonal antibodies prepared



-297-

against *mda-6* peptides) and actin (Oncogene Sciences Inc.) (Figs. 33 and 34). As observed with mRNA levels, no significant change in wild-type p53 protein occurs in H0-1 cells treated for 24 h with IFN- $\beta$ , MEZ or IFN- $\beta$  + MEZ (Fig. 33). In contrast, p21 mRNA and protein are induced in H0-1 cells, with IFN- $\beta$  + MEZ > MEZ > IFN- $\beta$  (Figs. 31A-E and 33). As seen with mRNA levels, the concentration of wild-type p53 protein decreases and p21 protein increases over a 96 h period in MEZ and IFN- $\beta$  + MEZ treated H0-1 cells (Fig. 34). Increases in p21 protein are also seen in H0-1 cells treated with IFN- $\beta$  for 48, 72 or 96 h, whereas no change in wild-type p53 protein occurs over the same period in similarly treated cells (Fig. 34). These results indicate that induction of p21 can occur without increases in wild-type p53 protein (IFN- $\beta$  treated cells) and elevated levels of p21 under conditions of residual growth arrest and/or terminal differentiation correlate with a reduction in wild-type p53 protein in H0-1 melanoma cells.

20

Inverse relationship between *mda-6* expression and evolution from normal melanocyte to metastatic melanoma

A prediction of applicants' melanoma aberrant differentiation model is that normal melanocytes should express elevated levels of specific *mda* genes with progressively less expression in primary and metastatic melanoma cells. As shown in Fig. 32, the level of *mda-6* is higher in an actively growing low-density SV40-transformed human melanocyte culture (FM516-SV) than in corresponding high-density proliferating human melanoma cells. The level of *mda-6* is variably increased in all of the melanomas treated with IFN- $\beta$  + MEZ (Fig. 32). Induction of *mda-6* occurs in logarithmically growing FM516-SV cells treated with IFN- $\beta$  + MEZ. However, only

-298-

the melanoma cells become terminally differentiated following growth for 96 h in IFN- $\beta$  + MEZ (data not shown). To evaluate *mda-6* levels as a function of melanoma evolution, *mda-6* and GAPDH (internal RNA expression standard) levels were determined by comparative RT-PCR in actively growing melanocytes (5 samples), a dysplastic nevus (1 sample), an SV40-transformed immortalized melanocyte culture (1 sample), RGP (1 sample) and early VGP (4 samples) primary melanomas and metastatic melanomas (6 samples) (Fig. 35). The highest levels of *mda-6* are found in actively growing melanocytes and the dysplastic nevus and the lowest relative levels of *mda-6* are present in primary and metastatic melanomas. The difference in relative *mda-6* expression (as a function of GAPDH expression) determined by comparative RT-PCR indicates that actively growing normal melanocytes express on average >4-fold more *mda-6* than actively growing metastatic melanoma cells ( $P < 0.01$ ). These results suggest an inverse correlation between levels of *mda-6* expression and human melanoma evolution.

Reduced expression of *mda-6* in Matrigel-progressed primary human melanoma cultures

The ability to study human melanoma progression by comparison of sequentially obtained cell lines established from the same patient is very limited since removal of the vast majority of RGP or thin VGP primary melanomas results in cure (Herlyn, 1990; Kerbel, 1990; Clark, 1991). Consequently, the derivation of genetically related variants from such tumors that express a biologically more aggressive phenotype must be obtained under experimental selections. One such method is "Matrigel-assisted" tumorigenic growth (Kobayashi et al.,

-299-

1994). For example, the early stage primary melanoma cell lines known as WM35, WM1341B and WM793 are non or poorly tumorigenic in nude mice in comparison to the great majority of advanced stage human melanomas (Kobayashi et al., 1994). However, co-injection of the early stage cell lines with Matrigel, a reconstituted basement membrane extract permits rapid tumor growth in nude mice and the derivation of sublines that will readily grow as solid tumors in secondary nude mouse recipients, even in the absence of Matrigel co-injection (Kobayashi et al., 1994). These tumorigenic Matrigel progressed sublines can be compared to the non/poorly tumorigenic parental cell lines for various properties such as relative *mda-6* expression. As shown in Figure 36, the level of *mda-6* as determined by RT-PCR in Matrigel progressed sublines of WM35, WM1341B and WM793 are variably reduced in comparison with the original patient-derived cell lines. The relative degree of suppression in *mda-6* is greatest in the WM793 series, that also display a more progressed phenotype as indicated by a low-level of *de novo* tumorigenic potential in nude mice in the absence of Matrigel (Kobayashi et al., 1994). The smallest reduction in expression occurs in the Matrigel-progressed WM35 RGP primary melanoma cells (Fig. 36). Treatment of parental and Matrigel-progressed RGP and early VGP melanomas with IFN- $\beta$  + MEZ results in increased *mda-6* expression (determined by Northern blotting) and growth suppression (data not shown). These observations provide further support for an inverse relationship between *mda-6* expression and human melanoma progression and melanoma growth.

Increased expression of *mda-6* in C8161 cells containing a normal human chromosome 6

-300-

Insertion of a normal human chromosome 6 into tumorigenic and metastatic C8161 human melanoma cells results in an extinction of the metastatic phenotype with a retention of tumorigenic potential (Table 5) (Welch et al., 1994). These results suggest that chromosome 6 contains a suppressor gene that can revert C8161 cells to a less-progressed stage in melanoma evolution (Welch et al., 1994). If *mda-6* expression correlates directly with states of melanoma progression, it would be predicted that actively growing C8161 cells should produce lower levels of *mda-6* than actively growing chromosome 6 containing C8161 cells. As anticipated, expression of *mda-6* increases in three independently derived chromosome 6 containing C8161 cells (Fig. 37). In addition, treatment of C8161 and neo6/C8161 hybrid clones with IFN- $\beta$  + MEZ for 96 hr results in growth suppression (Table 5) and increased *mda-6* mRNA expression (Fig. 37). These observations indicate a direct relationship between growth suppression and metastatic suppression in human melanoma cells and elevated expression of *mda-6* (p21).

-301-

Table 5: Properties of human melanoma cells containing a microcell-transferred chromosome 6

5	Cell Types <sup>1</sup>	Tumorigenicity <sup>2</sup>	Metastasis <sup>3</sup>
	C8161	+	+
	C8161/6.1 (neo6/C8161.1)	+	-
	C8161/6.2 (neo6/C8161.2)	+	-
10	C8161/6.3 (neo6/C8161.3)	+	-
15	=====		
20	Cell Types <sup>1</sup>	nm23 <sup>4</sup>	<u>mda</u> -6 <sup>5</sup> % growth inhibition <sup>6</sup>
	C8161	+/-	+/- 92
25	C8161/6.1 (neo6/C8161.1)	+++	+++ 67
	C8161/6.2 (neo6/C8161.2)	+ to ++	+++ 86
30	C8161/6.3 (neo6/C8161.3)	++	++ 89

<sup>1</sup>A neomycin-tagged normal human chromosome 6 was transferred into C8161 by microcell-mediated chromosome transfer as previously described (Welch et al., 1994). Retention of chromosome 6 in cell lines and tumor tissue was verified using PCR-RFLP with D6S87 and D6S37.

<sup>2</sup>Tumorigenicity was determined by injection of  $1 \times 10^6$  to  $1 \times 10^7$  cells subcutaneously or intradermally into the dorsolateral flank of 3-1/2 to 4-week old, female athymic nude mice (Harlan Sprague Dawley). +, palpable tumors form within 2 weeks after injection.

<sup>3</sup>Development of lung metastases following s.c., i.d. ( $1 \times 10^6$  cells) or i.v. ( $1 \times 10^6$  cells) injection of tumor cells. Spontaneous metastases were evaluated in the same mice in which tumorigenicity was assessed. At necropsy all organs were examined for grossly visible nodules. Lack of metastases was verified by histologic analysis of randomly submitted samples. Experimental metastases was measured in mice receiving a single

-302-

cell suspension of viable cells into the lateral tail vein.  
Table 1 Legend (Continued):

- 5   <sup>4</sup>Expression of nm23-H1 determined using Northern blot of total RNA (10-20  $\mu$ g) using the 0.9 kb fragment of nm23-H1 (Welch et al., 1994).
- 10   <sup>5</sup>Expression of *mda-6*/WAF1/CIP1 determined using Northern blot of total RNA (15  $\mu$ g) using the *mda-6* cDNA insert (Jiang & Fisher, 1993).
- 15   <sup>6</sup>Cell growth was determined by counting cells following 96 hr continuous treatment with IFN- $\beta$  (1000 units/ml) + MEZ (10 ng/ml). Results are the average percent growth inhibition versus untreated control cultures for triplicate plates that varied by  $\leq 10\%$  (Jiang et al., 1993).

20    Experimental Discussion

The specific genomic changes that mediate melanoma development and progression remain to be elucidated (Herlyn, 1990; Kerbel, 1990; Clark, 1991). To directly  
25   approach this question and to begin to identify and clone genes involved in growth control and differentiation in human melanoma cells applicants have used subtraction hybridization (Jiang & Fisher, 1993; Jiang et al., 1994a). cDNA libraries were constructed from untreated  
30   H0-1 human melanoma cells and these cDNAs were subtracted from cDNA libraries prepared from H0-1 cells treated with the combination of IFN- $\beta$  plus MEZ that induces an irreversible loss of proliferative ability and terminal differentiation (Fisher et al., 1985; Jiang & Fisher,  
35   1993; Jiang et al., 1993, 1994a). This approach has resulted in the identification of several novel *mda* cDNA clones that display enhanced expression as a function of growth suppression and induction of terminal differentiation in human melanoma cells (Jiang & Fisher,  
40   1993; Jiang et al., 1994a). In the present study applicants have analyzed *mda-6* (Jiang & Fisher, 1993;

-303-

Jiang et al., 1994a), whose open reading frame sequence (Fig. 30) is identical to the genes WAF1, CIP1 and SDI1 (El-Deiry et al., 1993; Harper et al., 1993; Noda et al., 1994). WAF1 was cloned using a strategy designed to  
5 identify inducible down-stream genes that are directly controlled by and might mediate the growth suppressing activity of the tumor suppressor gene p53 (El-Deiry et al., 1993). Introduction of WAF1 cDNA into human brain, lung and colon tumor cell cultures results in growth  
10 suppression (El-Deiry et al., 1993). In addition, WAF1 is induced by DNA damage in wild-type p53-containing cells and during the process of p53-associated G<sub>1</sub> arrest or apoptosis (El-Deiry et al., 1994). CIP1 was identified using an improved two-hybrid system and encodes a 21-kDa  
15 product that is a potent inhibitor of cyclin-dependent kinases (Harper et al., 1993). CIP1 induces growth suppression in normal diploid fibroblasts but only marginally inhibits growth in SV40-transformed diploid fibroblasts (Harper et al., 1993). SDI1 was identified  
20 and cloned from senescent human fibroblasts using an expression screening strategy designed to detect cDNAs that could prevent young fibroblasts from initiating DNA synthesis (Noda et al., 1994). The current studies indicate that *mda-6* (WAF1/CIP1/SDI1) expression is also  
25 related to growth regulation in human melanoma cells and its reduced expression may contribute to the progressive changes observed in the evolution of melanocytes into metastatic melanomas.

30 Cancer is a progressive disease often affected by the altered expression of oncogenes that promote the cancer phenotype and tumor suppressor genes that inhibit the cancer phenotype (for review see: Fisher, 1984; Bishop, 1991; Vogelstein & Kinzler, 1992; Lane, 1992). Recent

-304-

evidence indicates that the tumor suppressor gene p53 is a major component in the carcinogenic process (for review see: Vogelstein & Kinzler, 1992; Lane, 1992; Greenblatt et al., 1994). Inactivation of wild type p53 or expression of a mutant p53 phenotype has been found in a large number of human cancer subtypes (for review see: Vogelstein & Kinzler, 1992; Lane, 1992; Greenblatt et al., 1994). Intensive effort has been directed toward elucidating the mechanism by which wild type p53 regulates cell growth and prevents expression of the tumorigenic phenotype and the process by which p53 inactivation or mutagenic changes promote these processes. In this context, the identification of genes positively controlled by wild type p53, such as WAF1/CIP1, may prove important in defining the mechanism of action of this critical tumor suppressor gene and provide information about the process of tumor progression.

Metastatic human melanomas appear to be unique among human cancers in their low frequency of p53 mutations and the prevalence of wild-type p53 protein in advanced cancers (Volkenandt et al., 1991; Castresana et al., 1993; Greenblatt et al., 1994; Montano et al., 1994; Lu & Kerbel, 1994). Studies by Loganzo et al. (1994) show that metastatic melanoma cells contain two- to 20-fold more p53 protein, in the majority of samples representing wild-type p53, than do melanocytes. Similarly, a large proportion of the human melanoma cell lines presently analyzed also contain wild-type p53 protein. In normal melanocytes, the level of mda-6 (p21) is higher than in metastatic melanomas, even though metastatic melanoma may contain more p53 (Fig. 35) (Loganzo et al., 1994). The increased level of p53 protein in melanoma cells appears to be a consequence of stabilization of the protein,



-305-

i.e., the half-life is two- to five-fold greater than in melanocytes, irrespective of whether they contain wild-type or mutant-p53 (Loganzo et al., 1994). The stabilization of wt p53 protein in human melanoma cells does not result from the binding of this protein to either MDM2 or heat shock protein (Loganzo et al., 1994). The mechanism underlying this stabilization of wild-type p53 in metastatic melanomas is not presently known, but it might reflect a defective regulation of p53 that could allow these tumor cells to escape cell cycle arrest even in the presence of elevated p53. In fact, disturbances in p53 expression are a common occurrence in human melanomas and these abnormalities increase with progression (for review see: Lu & Kerbel, 1994). These findings suggest that melanoma may represent a novel malignancy, in that it can coexist and evolve to more aggressive stages even in the presence of elevated levels of nuclear localized wt p53 protein. However, it is also possible that the wild-type p53 protein in metastatic human melanoma cells is functionally inactive (perhaps by interacting with other melanoma proteins) or the wild-type p53 protein is normal, i.e., can both bind and transcriptionally activate target genes, but the downstream genes normally responsive to wild-type p53 are defective in metastatic human melanoma. The inability of wild-type p53 to elevate *mda-6* levels in metastatic melanoma, and consequently to induce proliferative control, could directly contribute to the increased instability of the evolving and progressing melanoma (Livingstone et al., 1992; Yin et al., 1992; Lu & Kerbel, 1994).

In human melanoma, *mda-6* is induced rapidly (within 15 min) and remains elevated following serum starvation as well as remaining elevated during terminal differentiation (Fig. 31A-E). In contrast, glioblastoma

-306-

multiforme cells blocked in G<sub>0</sub> by serum starvation or blocked in G<sub>1</sub> by mimosine treatment do not display increased levels of wild-type p53 or WAF1/CIP1 (El-Deiry et al., 1994). Induction of apoptosis after IL3 withdrawal, which also does not increase wild-type p53 levels, and DNA damage of cells containing a mutant p53 does not result in elevated levels of WAF1/CIP1 (El-Deiry et al., 1994). Recent studies suggest that wild-type p53 may not be obligatory for induction of WAF1/CIP1 (p21). Michieli et al. (1994) document a transient induction of WAF1/CIP1 following stimulation of growth arrested cells either containing or lacking wild-type p53 (fibroblasts from p53 knock out mice lacking p53 protein) with various growth factors. Jiang et al. (1994b) demonstrate that treatment of human promyelocytic leukemia HL-60 cells, which do not express p53, with agents inducing either granulocytic or macrophage/monocyte differentiation results in the rapid activation and persistent expression of mda-6 (p21). Steinman et al. (1994) also provide evidence that p21 is upregulated during induction of differentiation in a number of cell types, including hematopoietic and hepatoma cells, in a p53-independent pathway. All three of these studies also provide evidence that p21 is an immediate early response gene that is induced in the absence of protein synthesis.

The present study provides additional evidence indicating that induction of p21 expression is independent of wild-type p53 expression in human melanoma cells. An interesting, yet somewhat paradoxical observation, is the temporal decrease in wild-type p53 protein with a corresponding increase in p21 protein during the process of growth arrest and induction of terminal differentiation in H0-1 melanoma cells (Fig. 34). In a

-307-

number of cell culture model systems, p53 mRNA decreases as a function of growth suppression and the induction of differentiation (Shen et al., 1983; Mercer et al., 1984; Dony et al., 1985; Shobat et al., 1987; Khochbin et al., 5 1988; Richon et al., 1989; Hayes et al., 1991). Wild type p53 displays sequence-specific DNA-binding activity, sequence-specific transcriptional activation and induces growth suppression in a number of cell types, whereas all of these properties are lost in various mutant forms of the p53 protein (Ron, 1994; Pietenpol et al., 1994). The 10 reduced levels of *mda-6* (p21) in actively growing melanoma, even in the presence of high levels of wild type p53, and the elevations in p21 levels following wild type p53 suppression suggest that high levels of p21 expression may not be compatible with high levels of wild 15 type p53 in human melanoma. This may occur because wild type p53 is inducing a downstream gene that may directly or indirectly modify p21 expression. The induction of growth arrest and terminal differentiation program by IFN- $\beta$  + MEZ in H0-1 cells may result in genotypic changes 20 that mediate an inhibition of wild type p53 expression and consequently the absence of the downstream inhibitor of p21 expression. Alternatively, the wild type p53 protein that is present in metastatic melanoma may be functionally inactive or a downstream pathway modified by 25 wild type p53 may be aberrant in progressing melanoma cells. In this context, the inverse relationship observed between wild type p53 and p21 protein levels may be associated with but not functionally relevant to growth arrest and terminal differentiation induced by MEZ and 30 IFN- $\beta$  + MEZ.

In the present study applicants have not directly assayed for the effect of *mda-6* on progression in human melanoma. 35 However, experiments utilizing chromosome 6 containing

-308-

C8161 cells provide indirect evidence that *mda-6* (WAF1/CIP1/SDI1), which is located on chromosome 6p21.2 (El-Deiry et al., 1993), can directly modulate in vivo tumor growth and metastatic progression in human melanoma cells. Transfer of a normal chromosome 6 into the human melanoma cell lines UACC-903 and UACC-091 results in a suppression of tumorigenicity (Trent et al., 1990; Milikin et al., 1991), whereas microcell transfer of a normal chromosome 6 into the tumorigenic and metastatic human melanoma cell line C8161 results in a suppression of metastasis but a retention of tumorigenic potential (Welch et al., 1994). C8161 microcell hybrids (neo6/C8161) also display a small but significant increase in tumor latency time and have slower tumor growth rates in vivo than parental C8161 cells (Welch et al., 1994). However, even 30 additional weeks in animals does not result in metastatic lesions in mice injected with any of the three independent neo6/C8161 hybrids (Welch et al., 1994). In the present study, applicants demonstrate that C8161 cells contain lower levels of *mda-6* than three neo6/C8161 hybrids. Treatment with the combination of IFN- $\beta$  + MEZ results in enhanced expression of *mda-6* and growth suppression in parental C8161 and all three neo6/C8161 hybrid clones. These results strongly implicate *mda-6* as a potential mediator of growth control and metastatic progression in human melanoma cells. *mda-6* has now been cloned into the pMAMneo vector, that allows inducible expression of the inserted gene by dexamethasone (DEX) and which also contains a neomycin resistance gene permitting clonal isolation in G418 in the absence of *mda-6* expression (Jiang et al., in preparation). Electroporation of this gene into C8161 cells has resulted in the isolation of G418-resistant cultures containing the pMAMneo-*mda-6* construct. When grown in the presence of DEX, *mda-6* expression is induced

-309-

and growth is inhibited, whereas no growth suppression or *mda-6* expression occurs in the absence of DEX (Jiang et al., in preparation). These genetically modified C8161 cells, and melanoma cells containing an inducible antisense *mda-6* gene, will prove useful in directly determining the effect of *mda-6* expression on melanoma growth, differentiation and tumor progression.

Recent studies indicate that p21, the protein encoded by *mda-6* (WAF1/CIP1/SDI1), is a major contributor to many important cellular processes, including cell cycle regulation, cell growth, DNA repair and DNA replication (Xiong et al., 1992, 1993a, 1993b; Gu et al., 1993; Harper et al., 1993; El-Deiry et al., 1993, 1994; Waga et al., 1994; Li et al., 1994). p21 is a ubiquitous inhibitor of all cyclin-dependent kinases (Xiong et al., 1993b). The levels of p21 vary depending on the specific stage of the cell cycle and the interaction between p21 and specific cyclin-CDK enzymes appears to occur when these enzymes function in cell cycle control (Li et al., 1994). In the present study applicants demonstrate that *mda-6* (WAF1/CIP1/SDI1) expression inversely correlates with growth, differentiation and progression in human melanoma cells. These observations suggest that p21, that is encoded by *mda-6*, can affect cellular differentiation and neoplastic progression in human melanoma cells. The relative levels of *mda-6* are higher in growing human melanocyte and nevus cell lines than in RGP, VGP and metastatic melanomas, suggesting the possibility that expression of this suppressor protein may negatively regulate tumor progression. This possibility is supported by the observation that *mda-6* expression decreases in Matrigel-progressed early VGP melanomas and *mda-6* expression increases in chromosome 6 metastasis-suppressed C8161 melanoma cells. A direct relationship

-310-

between *mda-6* expression and melanoma growth and differentiation is indicated by the ability of IFN- $\beta$  + MEZ to induce growth suppression and with continuous exposure terminal differentiation in human melanoma cells. Apparently, increased levels of *mda-6* can be tolerated by human melanoma cells resulting in or correlating with growth arrest. However, the persistence of elevated levels of p21 in terminally differentiated human melanoma cells may be necessary to prevent cells from reentering the cell cycle, a mandatory requirement for terminal cell differentiation. In this context, identification of agent(s) that can increase *mda-6* (WAF1/CIP1/SDI1) expression in metastatic human melanoma cells may prove beneficial in the therapy of this malignancy by directly inducing an irreversible loss of proliferative capacity and terminal cell differentiation.

#### Materials and Methods

##### 20 Cell lines and culture conditions

H0-1 melanoma cells were established from a melanotic melanoma obtained from a 49-year-old female and were used between passage 125 and 160 (Fisher et al., 1985, 1986; Giovanella et al., 1976). FM516-SV is a normal human melanocyte culture immortalized by the SV40 T-antigen gene (Melber et al., 1989). Normal human melanocytes, FM713, FM723, FM741, FM841 and FM793, and a dysplastic nevus, N3153, were established from patients as described previously (Mancianti et al., 1988). WM35 was derived from an RGP primary human melanoma and WM278, WM1341B, WM793 and WM902B were derived from early VGP primary human melanomas (Herlyn, 1990; Herlyn et al., 1989). WM Matrigel progressed WM35, WM1341B and WM793 cells, referred to as P1-N1 and P2-N1 that indicates first and

-311-

second passage through nude mice injected with the appropriate cell type plus Matrigel, were developed as described (MacDougall et al., 1993; Kobayashi et al., 1994). C8161 is a highly metastatic amelanotic human melanoma cell line derived from an abdominal wall metastasis (Welch et al., 1991). C8161 clones containing a normal human chromosome 6, designated C8161/6.1 (neo6/C8161.1), C8161/6.2 (neo6/C8161.2) and C8161/6.3 (neo6/C8161.3), were established as described (Welch et al., 1994). Additional human melanoma cell lines isolated from patients with metastatic melanomas included F0-1, L0-1, SH-1, WM239 and WM239A (Giovanello et al., 1976; Fisher et al., 1985; Herlyn et al., 1989; Herlyn, 1990). Media and culture conditions used to grow the various cell types are described in the indicated references.

#### Cell growth and terminal cell differentiation assays

Cell growth and terminal differentiation assays were performed as described previously (Fisher et al., 1985, 1986; Jiang et al., 1993). Induction of terminal cell differentiation following 96 h growth in IFN- $\beta$  + MEZ was monitored by an irreversible loss of proliferative potential without a loss of cell viability (Fisher et al., 1985, 1986; Jiang et al., 1993). Briefly, cells were grown for 96 h in the presence of the various inducing agents, the inducers were removed and cultures were washed 3 X in medium without serum followed by the addition of inducer-free medium. Cultures were incubated for an additional 3, 6 and 10 d with a medium change without inducers every 3 d. Cell numbers were determined at d 4, 7, 10 and 14 after the beginning of the assay. Terminal differentiation was indicated by the absence of cell number increases and the retention of cell viability over the 7 to 14 d incubation in the absence of inducers.

-312-

Subtraction hybridization, RACE and sequence analysis

Identification and cloning of *mda-6* by subtraction  
5 hybridization was achieved as described (Jiang & Fisher,  
1993). A full-length *mda-6* cDNA was isolated by screening  
a differentiation inducer-treated H0-1 cDNA library  
(Jiang & Fisher, 1993) and using the procedure of rapid  
10 amplification of cDNA ends (RACE) as described (Frohman  
et al., 1988; Loh et al., 1989; Ohara et al., 1989).  
Sequence analysis was determined as described (Sanger et  
al., 1977; Su et al., 1993). The complete sequence of  
*mda-6* consists of 2149 nucleotides (U09579 in GenBank)  
and the longest open reading frame starting with a  
15 methionine codon at position 95 in the nucleotide  
sequence encodes a 164-amino acid polypeptide. Compared  
with the current protein database releases using the  
GCG/TFASTA program, the predicted *mda-6* amino acid  
sequence is identical to the sequences of WAF1/CIP1/SDI1  
20 (El-Deiry et al., 1993; Harper et al., 1993; Noda et al.,  
1994).

RNA Isolation, Northern Blotting and RT-PCR

25 Total cytoplasmic RNA was isolated and Northern blotting  
hybridization was performed as described (Reddy et al.,  
1991; Su et al., 1991; Jiang et al., 1992). Ten  $\mu$ g of RNA  
was denatured with glyoxal/DMSO, electrophoresed on 1.0%  
agarose gels, transferred to nylon membranes and  
30 hybridized to a  $^{32}$ P-labeled p53 probe (Baker et al.,  
1990). The nylon membrane was stripped and hybridized to  
a  $^{32}$ P-labeled *mda-6* probe (Jiang and Fisher, 1993) and  
then after a second stripping the membrane was hybridized  
to a  $^{32}$ P-labeled rat GAPDH probe (Fort et al., 1985), as



-313-

described previously (Reddy et al., 1991; Su et al., 1991; Jiang et al., 1992). Following hybridization, the filters were washed and exposed for autoradiography (Reddy et al., 1991; Su et al., 1991; Jiang et al., 1992). *mda-6* and GAPDH gene expression were also determined by reverse transcription-polymerase chain reaction (RT-PCR) as described (Adollahi et al., 1991; Lin et al., 1994; Jiang et al., 1994b). Total cytoplasmic RNA was treated with 0.5 units DNase (Boehringer-Mannheim Biochemicals)/ $\mu$ g RNA in 15% glycerol, 10 mM Tris, pH 7.5, 2.5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 80 mM KCl, 1 mM CaCl<sub>2</sub> and 1 unit/ml RNasin (Promega) at 30°C for 10 min. RNA was extracted with phenol-chloroform, precipitated with sodium acetate/ethanol and RNA pellets were resuspended in diethylpyrocarbonate-treated H<sub>2</sub>O. One  $\mu$ g of total RNA was reverse transcribed with 200 units of murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) in 20  $\mu$ l containing 1 mM deoxyribonucleotide triphosphates, 4 mM MgCl<sub>2</sub>, 10 mM Tris, pH 8.3, 50 mM KCl, 0.001% gelatin and 0.2  $\mu$ g oligo-dT primer. Samples were adjusted to 100  $\mu$ l with buffer containing 0.2 mM deoxyribonucleotide triphosphates, 2 mM MgCl<sub>2</sub>, 10 mM Tris, pH 8.3, 50 mM KCl and 0.001% gelatin. Fifty pmol of each primer, 1.5 units Taq DNA polymerase (Perkin-Elmer Cetus) were added and samples were covered with mineral oil, heated at 95°C for 5 min and subjected to 25 cycles of PCR in a Perkin-Elmer Thermal Cycler using 1 min denaturation at 94°C, 2 min annealing at 55°C and 3 min polymerization at 72°C. After extraction with chloroform, 20  $\mu$ l of products were electrophoresed, blotted onto nylon filters and hybridized with an *mda-6* or GAPDH specific probe. The *mda-6* primers were 5' to 3' CTCCAAGTACACTAAGCACT and TAGTTCTACCTCAGGCAGCT (corresponding to nt 1527 to 1546) (GenBank accession

-314-

number U09579); and the GAPDH primers were 5' to 3'  
TCTTACTCCTTGGAGGCCATG and CGTCTTCACCACCACCATGGAGAA  
(corresponding to nt 1070 to 1053) (Tokunaga et al.,  
1987).

5

#### Immunoprecipitation analyses

Immunoprecipitation analyses were performed as described  
previously (Duigou et al., 1991; Su et al., 1993; Jiang  
et al., 1994b). Logarithmically growing H0-1 cells were  
either untreated or treated for 24, 48, 72 or 96 h with  
IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml) or IFN- $\beta$  + MEZ  
(2000 units/ml + 10 ng/ml) in 10-cm plates. Cultures were  
starved of methionine for 1 h at 37°C in methionine-free  
medium, cells were concentrated by pelleting and labeled  
for 1 h (p53) or 4 h (p21 and Actin) at 37° in 1 ml of the  
same medium with 100  $\mu$ Ci of [ $^{35}$ S] (NEN; Express  $^{35}$ S). After  
labeling, the cells were washed twice with ice-cold  
phosphate-buffered saline and lysed for 1 h on ice by the  
addition of RIPC (20 mM Tris-base, pH 7.5, 500 mM NaCl,  
0.05% Nonidet P-40, 100  $\mu$ g/ml phenylmethylsulfonyl  
fluoride and 0.02% sodium azide). The lysate was  
clarified by centrifugation in an Eppendorf microfuge at  
10,000 X g for 10 min at 4°C. H0-1 samples containing 4  
X 10<sup>6</sup> counts were incubated with 2  $\mu$ g of p53 monoclonal  
antibody (Ab1; PAb421) (Oncogene Sciences), WAF1/CIP1 (C-  
19) (Santa Cruz Biotechnology) (or MDA-6 peptide-derived)  
rabbit polyclonal IgG or actin monoclonal antibody  
(Oncogene Sciences) with rocking at 4°C for 24 h. Labeled  
cell lysates were also prepared from F0-1, L0-1, SH-1,  
WM239, FM516-SV, SW480, MeWo, human skin fibroblasts and  
Saos-2 cells. Samples containing 4 X 10<sup>6</sup> counts were  
incubated with 2  $\mu$ g of the p53 monoclonal antibody Ab1  
(PAb421) or Ab3 (PAb240) (Oncogene Sciences). The next

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-315-

day, 30  $\mu$ l (packed volume) of protein G-agarose (Oncogene Sciences) was added to each tube, and incubation with rocking at 4°C continued for another hour. The protein G pellets were then washed five times with 1 ml of ice-cold

5    RIPC:phosphate-buffered saline (1:1, v/v). Thirty  $\mu$ l of sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer were added to the pellets, and the sample was heated at 87°C for 3 min. The samples were loaded onto an

10    10% polyacrylamide gel and run overnight at 40 V. The gel contained Rainbow protein markers (Amersham) for sizing. Gels were fixed with 10% acetic acid plus 10% methanol for 30 min, incubated in DMSO for 30 min, incubated with 10% 2,5-diphenyloxazole in DMSO for 30 min, washed three times with cold water (10 min each), dried, and exposed

15    to film.

-316-

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-323-

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-324-

Eighth Series of ExperimentsProperties of *mda-7*

5

*mda-7* is a novel cDNA (it has no sequence homology with previously reported genes in the various DNA data bases). The full-length cDNA contains 1718 nt, the open reading frame extends from nt 275 to nt 895 and encodes a protein of 206 amino acids, containing a membrane domain and three potential glycosylation sites.

10

Expression in H0-1 Human Melanoma Cells

Increased expression of *mda-7* after 24 hr treatment of H0-1 cells with recombinant human fibroblast interferon (IFN- $\beta$ ) (2000 units/ml), MEZ (10 ng/ml) and to the greatest extent with IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml).

20

Increased expression of *mda-7* is observed in H0-1 cells treated for 96 hr with IFN- $\beta$  (2000 units/ml), MEZ (10 ng/ml), MPA (3  $\mu$ M), IFN- $\beta$  + IFN- $\gamma$  (1000 units/ml + 1000 units/ml), IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml, MPA + MEZ (3  $\mu$ M + 10 ng/ml) and RA + MEZ (2.5  $\mu$ M + 10 ng/ml). Maximum induction is observed with IFN- $\beta$  + MEZ followed by MPA + MEZ and IFN- $\beta$  + IFN- $\gamma$ .

25

The relative level of *mda-7* induction correlates with the degree of growth suppression observed H0-1 cells treated with the various growth and differentiation modulating agents. The greatest increase in expression is observed in cells induced to irreversibly lose proliferative capacity and become terminally differentiated by treatment with IFN- $\beta$  + MEZ.

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-325-

Expression in Additional Human Melanoma Cells

Increased expression of *mda-7* occurs in H0-1, C8161, C8161/6.3 (a C8161 human melanoma cell clone containing  
5 an inserted normal human chromosome 6: These cells are tumorigenic in nude mice, but unlike parental C8161 cells they are non-metastatic), F0-1, L0-1, SH-1, WM278 and WM239 human melanoma cells treated with IFN- $\beta$  + MEZ for 24 hr. This gene is constitutively expressed in  
10 immortalized human melanocytes FM5169 (transformed by SV40). However, no increase in expression is observed in FM5169 following IFN- $\beta$  + MEZ treatment for 24 hr.

*mda-7* is either variably expressed or variably induced in  
15 all human melanoma cells treated with IFN- $\beta$  + MEZ. In contrast, although this gene is expressed in melanocytes, no change in expression is observed following a 24 hr treatment with IFN- $\beta$  + MEZ.

20 Expression in Human Neuroblastoma Cells

*mda-7* is not expressed in LAN human neuroblastoma cells as determined by reverse transcription-polymerase chain reaction (RT-PCR). *mda-7* expression is not induced by  
25 treatment with RA for 5 days, but it is induced after 5 days growth in the medium containing phenylacetate or the combination of phenylacetate and RA.

*mda-7* is induced in human neuroblastoma cells as a  
30 function of growth arrest and induction of differentiation. Expression of *mda-7* may contribute to growth arrest and terminal differentiation in human neuroblastoma cells.

35 Expression in Human Promyelocytic Leukemia Cells (HL-60)

-326-

and Histiocytic Lymphoma (U-937) Cell Lines

mda-7 expression is not detected in HL-60 and U-937 cells using RT-PCR. mda-7 expression is induced in HL-60 and  
5 U-937 cells following treatment with the growth suppressing and differentiation inducing agent TPA. mda-7 expression persists in terminally differentiated HL-60 cells after treatment with TPA for 2 days and RA for 4 days.

10 mda-7 is induced during differentiation along both granulocytic and agranulocytic (monocytic/macrophage) lineages in human promyelocytic leukemia and histiocytic lymphoma cells. Expression of mda-7 may contribute to  
15 the growth arrest and terminal differentiation in hematopoietic cells.

Expression in Senescent Human Cells

20 mda-7 expression is not detected using RT-PCR in IMR90 human cells displaying proliferative potential (i.e., non-senescent cells). mda-7 expression is detected in IMR90 cells grown for extended times in culture (OLD) and approaching senescence.

25 mda-7 gene expression inversely correlates with proliferative potential in human cells. mda-7 expression is activated during cellular senescence. This gene may contribute to proliferative capacity in cells and may  
30 function as a genetic marker and/or regulatory switch of cellular senescence.

Expression in Normal Cerebellum, a Central Nervous System Tumor (Glioblastoma Multiforme) (GBM) and Normal Skin  
35 Fibroblast Cell Lines

-327-

mda-7 is not expressed de novo in normal cerebellum, GBM or normal skin fibroblasts. Expression of mda-7 is induced in normal cerebellum, GBM and normal skin fibroblasts following a 24 hr treatment with IFN- $\beta$  + MEZ.

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mda-7 is not expressed de novo, but it is susceptible to induction by IFN- $\beta$  + MEZ in human cerebellum, GBM and normal human skin fibroblasts.

10     Expression in Colorectal (SW613), Endometrial Adenocarcinoma (HTB113) and Prostate Carcinoma (LNCaP)

mda-7 is expressed de novo in colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) or prostate carcinoma (LNCaP). mda-7 is not induced in colorectal carcinoma (SW613), endometrial adenocarcinoma (HTB113) or prostate carcinoma (LNCaP) cells following a 24 h treatment with IFN- $\beta$  + MEZ.

20     This gene is neither expressed de novo nor inducible by IFN- $\beta$  + MEZ human carcinomas.

Effect of Various Treatment Protocols on Expression in H0-1 Cells

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Treatment with IFN- $\beta$  (2000 units/ml; 24 h), MEZ (10  $\mu$ g/ml; 24 h), IFN- $\beta$  + MEZ (2000 units/ml + 10 ng/ml; 24 h and 96 h), IFN- $\alpha$  + MEZ (2000 units/ml + 10 ng/ml; 24 h), adriamycin (0.1 ng/ml; 24 h), vincristine (0.1  $\mu$ g/ml; 24 h), and UV (10 joules/mm<sup>2</sup> and assayed 24 h later) results in increased mda-7 expression in H0-1 cells. mda-7 is also induced after 96 h treatment with MPA (2  $\mu$ M), IFN- $\beta$  + IFN- $\gamma$  (1000 units/ml + 1000 units/ml), MPA + MEZ (3  $\mu$ M + 10 ng/ml) and RA + MEZ (2.5  $\mu$ M + 10 ng/ml).

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-328-

Highest level of expression observed in H0-1 cells treated with IFN- $\beta$  + MEZ for 24 or 96 h.

No induction in *mda-7* expression is observed in H0-1 cells treated with IFN- $\alpha$  (2000 units/ml; 24 h), IFN- $\gamma$  (2000 units/ml; 96 h), phenyl butyrate (4 mM PB for 24 h, 4d or 7d), cis-platinum (0.1  $\mu$ g/ml; 24 h), gamma irradiation (treated with 3 gray and analyzed after 24 h), actinomycin D (5  $\mu$ g/ml for 2 h, assayed 24 h later), TNF- $\alpha$  (100 units/ml; 24 h) or VP-16 (5  $\mu$ g/ml; 24 h).

#### General Conclusions

*mda-7* is a growth, differentiation-regulated and senescence-associated novel gene which displays the following properties: 1) it is inducible during terminal differentiation (treatment with IFN- $\beta$  + MEZ for 96 h) and following treatment for 96 h with many growth modulating and differentiation inducing agents; 2) treatment for 24 h with IFN- $\beta$  + MEZ results in increased expression in all human melanomas tested, but not in an SV40-immortalized human melanocyte; 3) it is not expressed in growing human neuroblastoma cells but it is inducible following growth suppression and the induction of terminal differentiation 4) it is not expressed in human promyelocytic leukemia (HL-60) and human histiocytic lymphoma (U-937) cells but it is induced following the induction of growth arrest and terminal differentiation; 5) it is not expressed in actively growing human cells but it is induced during cellular senescence; 6) it is not expressed de novo but it is highly inducible by IFN- $\beta$  + MEZ within 24 h in normal cerebellum, GBM and normal skin fibroblast cells; 7) it is not expressed or inducible in colorectal, endometrial or prostate carcinomas; and 8) increased expression is induced in H0-1 cells treated with



-329-

adriamycin, vincristine and UV irradiation.

*mda-7* is a novel growth, terminal differentiation- and senescence-regulated gene displaying increased expression in all melanomas (but not in melanocytes), and in normal skin fibroblasts and in both normal cerebellum and GBM cells treated with IFN- $\beta$  + MEZ. *mda-7* is not expressed de novo, but it is induced during growth arrest and differentiation in human neuroblastoma, leukemia and histiocytic lymphoma cells. *mda-7* is not expressed in growing nonsenescent cells but it is expressed in senescent cells. In contrast, *mda-7* is not expressed or induced in a series of carcinomas. *mda-7* may be useful; 1) as a marker for specific tissue lineage's (i.e., melanomas from keratinocytes) (diagnostic applications); 2) in distinguishing fibroblasts (inducible with IFN- $\beta$  + MEZ) from carcinomas (non-inducible with IFN- $\beta$  + MEZ) (diagnostic applications); 3) in identifying cells that have lost proliferative capacity and become senescent; 4) in monitoring induction of differentiation in cancer cells resulting during the differentiation therapy of cancer; 5) for the identification of agents capable of inducing growth suppression and various components of the differentiation process (including terminal differentiation) in human melanomas, neuroblastomas, leukemias and lymphomas (drug screening programs to identify new differentiation-inducing and chemotherapeutic agents); and 6) distinguishing melanocytes, and perhaps nevi, from early and late stage melanoma cells (diagnostic applications). This gene (used in a sense orientation in an appropriate expression vector) may also prove useful in inhibiting growth and inducing terminal differentiation in human melanomas (therapeutic applications). When used in an antisense orientation and inserted into bone marrow cells this gene

-330-

might prevent damage resulting from chemotherapy and radiation (therapeutic applications).

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Fisher, Paul B.  
Jiang, Hongping
- (ii) TITLE OF INVENTION: METHOD FOR GENERATING A  
SUBTRACTED CDNA LIBRARY AND USES OF THE GENERATED  
LIBRARY
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: John P. White, c/o Cooper & Dunham
  - (B) STREET: 30 Rockefeller Plaza
  - (C) CITY: New York
  - (D) STATE: New York
  - (E) COUNTRY: U.S.A.
  - (F) ZIP: 10112
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: US
  - (B) FILING DATE: 25-OCT-1993
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
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  - (B) REGISTRATION NUMBER: 28,678
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 158 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

-331-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGGACTTGTG TTCTGACTAG AACTCAACAT GTTACTAGGC ACATGTGTCA TGTCTCAGGT 60  
 CAGTGCTGTG ACAGAATTGA TACGAGAGAA ATGTCGCTTA TGCTATCACT GATCTACACA 120  
 TGTCTGATAG ATAGTCAGAT ACAGATGATG AGGAATCT 158

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 270 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAATTCAGTG AACTCTTTTC TCATTCTCTT TGTTTTGTGG CACTTCACAA TG TAGAGGAA 60  
 AAAACCAAAT GACCGCACTG TGATGTGAAT GGCACCGAAG TCAGATGAGT ATCCTGTAGG 120  
 TCACCTGCAG CCTGGCTTGC CACTTGTCTT AACTCTGAAT ATTCATTTC AAAGGTGCTA 180  
 AAATCTGAAA TCTGCTAGTG TGAAGTTGCT CTACTCTCTG AATGATTCAA TCCTATTCAT 240  
 ACTATCTTGT AGATATATCA ACTAAAAAAA 270

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 245 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCCTCCTCTG CACCATGGCT CTCTGCAACC AGTTCTCTGC ATCACTTGCT GCTGACACGC 60  
 CGACCGCCTG CTGCTTCAGC TACACCTCCC GGCAGATTCC ACAGAATTTC ATAGCTGACT 120  
 ACTTTGAGAC GAGCAGCCAG TGCTCCAAGC CCGGTGTCAT CTTCTAACC AAGCGAACCG 180  
 GGCAGGTCTG TGCTGACCCC AGTGAGGAGT GGGTCCAGAA ATATGTCAGC GACCTGGAGC 240

- 332 -

TGAGT

245

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 245 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCCTCCTCTG CACCATGGCT CTCTGCAACC AGTTCTCTGC ATCACTTGCT GCTGACACGC 60  
CGACCGCCTG CTGCTTCAGC TACACCTCCC GGCAGATTCC ACAGAATTTT ATAGCTGACT 120  
ACTTTGAGAC GAGCAGCCAG TGCTCCAAGC CCGGTGTCAT CTTCTAACC AAGCGAAGCC 180  
GGCAGGTCTG TGCTGACCCC AGTGAGGAGT GGGTCCAGAA ATATGTCAGC GACCTGGAGC 240  
TGAGT 245

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 144 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTCTTCTTTG TAAAAGTTTT TAATACACTG CTGAAAGATA AATTCATTCC AAAGAGAATA 60  
ATTATATAGC AAGATATTAT CGGCACAGTG GTTCTTAGA GGTAAATAGC GCCTCACGTG 120  
TGTTAGATGC TGAATCTGAC CAAA 144

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 193 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

-333-

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
CTGCAAAAGA AGTGTGCCGA CTATAAATAA ATGGTGAAAT CATCTGCAA TGTGGCCAGG    60
CTTGGGGAAC AATGATGGTG CACAAAGGCT TAGATTTGCC TTGTCTCAA ATAAGGAATT    120
TTGTAGTGGT TTCAAATAT CACAAGAACG TACAAGTGGT AGATACTATC ACATTCACTG    180
ACTATCAGAG TCG                                                         193
```

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 301 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ACAAACCACT GATTCCCCCTT CCTCAGATAC TGGGACTAAC AGCTTCACCT GGTGTTGGAG    60
GGGCCACGAA GCAAGCCAAA GCTGAAGAAC ACATTTTAAA ACTATGTGCC TATCTTGATG    120
CATTTACTAT TAAAACTGTT AAAGAAAACC TTGATCAACT GAAAAACCA ATACAGGAGC    180
ATGCAAGAAG TTTGCCATTG CAGATGCAAC CAGAGAAGAT CCATTTAAAG AGAAACTTCT    240
AGAAATAATG ACAAGGATTC AACTTATTG TCAAATGAGT CCAATGTCAG ATTTTGGACT    300
C                                                                      301
```

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 218 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

-334-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

ATGCCACGTG GGCTCATATG GGGCTGGGAG TAGTTGTCTT TCCTGGCACT AACGTTGAGC   60
CCCTGGAGGC ACTGAAGTGC TTAGTGACTT TGGAGTATTG GGGTCTGACC CAAACACCTT   120
CCAGCTCCTG TAACATACTG GCCTGGACTG TTTTCTCTCG CGCCTCCCCA TGTGCTCCTG   180
GTTCCCGTTT CCTCCACCTA GACTGTAAAC CTCTCGCA                             218

```

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 279 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```

CCTGCAGTCC TGAAGCGCG AGGGCCTCAA ACGCGCTCTA CATCTTCTGC CTTAGTCTCA   60
GTTTGCCTGT CTTAATTATT ATTTGTGTTT TAATTAAAC ACCTCCTCAT GTACATACCC   120
TGGCCGCCCC CTGCCCCCA GCCTCTCGGA TTAGAATTAT TTAAACAAAA ACTAGGCGGT   180
TGAATGAGAG GTTCTATGA GTACTGGCA TTTTATTTT ATGAAATACT ATTTAAAGCC   240
TCCTCATCCC ATGTTCTCCT TTTCCTCTCT CCCGGAGTT                             279

```

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 193 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```

CAGAATATTG TGCCCCATGC TTCTTTACCC CTCACAATCC TTGCCACAGT GTGGGCAGTG   60
GATGGGTGCT TAGTAAGTAC TTAATAAACT GTGGTGCTTT TTTTGGCCTG TCTTTGGATT   120
GTAAAAAAC AGAGAGGGAT GCTTGGATGT AACTGAACT TCAGAGCATG AAATCACACT   180

```

-335-

GTCTCTGATA TCT

193

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 182 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTAAAGTTTG CCCTTGTGCT AAAGTGCCAG TGTATGTATG TTATACTTGA TTTGGTTGTA 60  
AACTATATTT CAAAGTAAAC CCTAGTGTA TAAGTTTTAT AACTAAAAAG GTTTAAGCTG 120  
CTAAAACTAT TTTTAAGAGA TGTGAAATCG AGTATGGGAC TATCTTTTTT TCCTCCTCTA 180  
AA 182

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 216 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AAAACTTTCA AGAGATTTAC TGACTTTCCT AGAATAGTTT CTCTACTGGA AACCTGATGC 60  
TTTTATAAGC CATTGTGATT AGGATGACTG TTACAGGCTT AGCTTTGTGT GAAAACCACT 120  
CACCTTTCTC CTAGGTAATG AGTAGTGCTG TTCATATTAC TTTAGTTCTA TAGCATACTC 180  
GATCTTTAAC ATGCTATCAT AGTACATTAG ATGATG 216

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 257 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- 336 -

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGCACGTCAC CCACCTTCCG GCGGCCGAAG ACGTGCAC TCCGGAGACA GCCCAAATAT	60
CCTCGGAAGA GCGCTCCCAG GAGAAACAAG CTTGACCACT ATGCTATCAT CAAGTTTCCG	120
CTGACCACTG AGTCTGCCAT GAAGAAGATA GAAGACAACA ACACACTTGT GTTCATTGTG	180
GATGTTAAAG CCAACAAGCA CCAGATTAAC AGCTGTGAGA GCTGTATGAC ATTGATGTGC	240
AGTACACCTG ATCGTCT	257

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 241 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TAAAAAAATT CATTCTCTGT GGTATCCAAG AATCAGTGAA GATGCCAGTG AAACCTCAAG	60
CAAATCTACT TCAACACTTC ATGTATTGTG TGGGTCTGTT GTAGGGTTGC CAGATGCAAT	120
ACAAGATTCC TGGTTAAATT TGAATTTTCA TAAACAATGA ATAGTTTTTC ATTGTACATG	180
AAATATCAGA ACATACTTAT ATGTAAGTAT ATTATTGATG ACAAACACAA TATTTAATAT	240
A	241

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 177 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO



- 337 -

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGGGGTGAAA CTTTCCAGTT TACTGAACTC CAGACCATGC ATGTAGTCCA CTCCAGAAAT 60  
CATGCTCGCT TCCTTGGCAC ACAGTGTCTT CCTGCCAAAT GACCCTAGAC CCTCTGTCCT 120  
GCAGAGTCAG GGTGGCTTTT ACCCTGACTG TGTCGATGCA GAGTCTGCTC GACAGAT 177

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 143 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TACGATCAGA CTGTTACATT TAGCAATCAA CAGCATGGGG CGAAAAAAAA AAATCTACTT 60  
AAAACCCCTTT GTTGAATGC TTTACACTTT CCACAGAACA GAAACTAAAA TAACTGTTTA 120  
CATTAGTCAC AATACAGTCT CGA 143

-338-

What is claimed is:

1. A method of generating a subtracted cDNA library of a cell comprising:
  - a) generating a cDNA library of the cell;
  - b) isolating double-stranded DNAs from the cDNA library;
  - c) releasing the double-stranded cDNA inserts from the double-stranded DNAs;
  - d) denaturing the isolated double-stranded cDNA inserts;
  - e) hybridizing the denatured double-stranded cDNA inserts with a labelled single-stranded nucleic acid molecules which are to be subtracted from the cDNA library; and
  - f) separating the hybridized labeled single-stranded nucleic acid molecule from the double-stranded cDNA inserts, thereby generating a subtracted cDNA library of a cell.
2. A method of claim 1, wherein the cDNA library allows propagation in single-stranded circle forms.
3. A method of claim 2, wherein the cDNA library is a  $\lambda$ ZAP cDNA library.
4. A method of claim 1, wherein the releasing of the double-stranded cDNA is performed by digestion of appropriate restriction enzyme.
5. A method of claim 1, wherein the denaturing of step d) is by boiling.
6. A method of claim 1, wherein the single-stranded

-339-

nucleic acid molecules are DNAs.

7. A method of claim 1, wherein the single-stranded nucleic acid molecules are labelled with biotin.
8. A method of claim 7, wherein the separating of step f) is performed by extraction with streptavidin-phenol: Chloroform.
9. A method of claim 1, wherein the single-stranded nucleic acid molecules are from another cDNA library.
10. A method of claim 9, wherein the cDNA library allows propagation in single-stranded circle forms.
11. A method of claim 10, wherein the cDNA library is a  $\lambda$ ZAP cDNA library.
12. A method of claim 11, wherein the cell is an HO-1 melanoma cell treated with IFN- $\beta$  and MEZ.
13. A method of claim 12, wherein the single-stranded nucleic acid molecules are from another cDNA library of a HO-1 melanoma cell.
14. A method of claim 10, wherein the cell is terminally differentiated and the single-stranded nucleic acid molecules are from another cDNA library of an undifferentiated cell.
15. A method of claim 10, wherein the cell is undifferentiated and the single-stranded nucleic acid molecules are from another cDNA library of a terminally differentiated cell.

-340-

16. A method of claim 14, wherein the cell is selected from a group consisting essentially of neuroblastoma cell, glioblastoma multiforme cell, myeloid leukemic cell, breast carcinoma cell, colon carcinoma cell, endometrial carcinoma cell, lung carcinoma cell, ovarian carcinoma cell and prostate carcinoma cell.
17. A method of claim 10, wherein the cell is induced to undergo reversible growth arrest or damage and the single-stranded nucleic acid molecules are from another cDNA library of an uninduced cell.
18. A method of claim 10, wherein the cell is an uninduced cell and the single-stranded nucleic acid molecules are from a cell induced to undergo reversible growth arrest or damage.
19. A method of claim 10, wherein the cell is at one developmental stage and the single-stranded nucleic acid molecules are from another cDNA library of the cell at a different developmental stage.
20. A method of claim 10, wherein the cell is cancerous and the single-stranded nucleic acid molecules are from another cDNA library from a normal cell.
21. A method of claim 10, wherein the cell is from the breast, brain, meninges, spinal cord, colon, endometrium, lung, prostate and ovary.
22. A method of claim 10, further comprising introducing the subtracted library into host cells.
23. A subtracted library generated by the method of

-341-

claim 1.

24. A subtracted library generated by the method of claim 13.
25. A method of identifying a melanoma differentiation associated gene comprising:
  - a) generating probes from clones of the subtracted library of claim 24; and
  - b) hybridizing the probe with the total RNAs or mRNAs from H0-1 cells treated with IFN- $\beta$  and MEZ and the total RNAs or mRNAs from untreated H0-1 cells, hybridization of the probe with the total RNAs or mRNAs from the treated H0-1 cell but no or reduced hybridization with the total RNAs or mRNA from untreated cells indicating that the clone from which the probe is generated carries a melanoma differentiation associated gene.
26. A gene identified by the method of claim 25.
27. An isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-1*.
28. A cDNA of claim 27.
29. An isolated nucleic acid molecule of claim 27, wherein the protein is a human protein.
30. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence

-342-

of the nucleic acid molecule of claim 27.

31. An isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-4*.
32. A cDNA of claim 31.
33. An isolated nucleic acid molecule of claim 31, wherein the protein is a human protein.
34. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of claim 31.
35. A method of detecting the expression of *mda-4* gene in a cell comprising:
  - a) isolating the nucleic acids in the cell;
  - b) hybridizing the isolated nucleic acids with the nucleic acid molecules of claim 34 under conditions permitting hybrids formation; and
  - c) detecting hybrids formed, the detection of the hybrids indicating the expression of *mda-4* gene in the cell.
36. A method to indicate the tissue lineage of a cell comprising detecting the expression of *mda-4* gene using the method of claim 35, the expression of *mda-4* gene indicating that the tissue lineage of the cell is neuroectodermal.

-343-

37. A method to monitor response to DNA damage induced by gamma irradiation and UV irradiation of a cell comprising hybridizing the nucleic acids from the cell with the nucleic acid molecule of claim 34, hybridization of the nucleic acid from the cell indicating that there is response to the DNA damage of the cell.
38. A method of monitoring response to treatment with chemotherapeutic agents which work in a similar manner as cis-platinum in a cell comprising hybridizing the nucleic acids from the cell with the nucleic acid molecule of claim 34, hybridization of the nucleic acid from the cell indicating that the cell responds to the treatment with the chemotherapeutic agents.
39. A method for detecting types I or II interferons in a sample comprising:
  - a) incubating the sample with a target cell containing the 5' regulatory element of *mda-4* permitting binding of the type I or type II interferons transcriptional regulatory proteins to the 5' regulatory elements; and
  - b) detecting the binding indicating the presence of type I or type II interferons.
40. A method of claim 39, wherein the cell is a eukaryotic cell.
41. A method of claim 39, wherein the 5' regulatory element is linked to the native *mda-4* gene and the detection of binding is by examination of the

-344-

elevated expression of the *mda-4* gene.

42. A method of claim 39, wherein the 5' regulatory element is linked to a marker gene.
43. A method of claim 42, wherein the marker gene is  $\beta$ -galactosidase, luciferase or CAT.
44. A method for detecting types I or II interferons in a sample comprising:
  - a) incubating the sample with the 5' regulatory element of the *mda-4* gene under conditions permitting binding of types I and II interferons transcriptional regulatory proteins to the regulatory elements; and
  - b) detecting the binding of the types I or II interferons transcriptional regulatory proteins to the regulatory elements, the binding indicating the presence of type I or type II interferons.
45. A method for identifying compound capable of inducing terminal differentiation in human melanoma cells comprising:
  - a) incubating appropriate concentration of the human melanoma cells with an appropriate concentration of the compound; and
  - b) detecting the expression of *mda-4* using the method of claim 35, the expression of *mda-4* gene indicating that the compound is capable of inducing terminal differentiation in human



-345-

melanoma cells.

46. An isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-5*.
47. A cDNA of claim 46.
48. An isolated nucleic acid molecule of claim 47, wherein the protein is a human protein.
49. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of claim 46.
50. A method of detecting the expression of *mda-5* gene in a cell comprising:
  - a) isolating the nucleic acids in the cell;
  - b) hybridizing the isolated nucleic acids with the nucleic acid molecules of claim 49 under conditions permitting hybrids formation; and
  - c) detecting hybrids formed, the detection of hybrids indicating the expression of *mda-5* gene in the cell.
51. A method for distinguishing a normal neuroectodermal cell from a malignant neuroectodermal cell comprising detecting the expression of *mda-5* gene using the method of claim 50, the expression of *mda-5* gene indicating that the cell is normal neuroectodermal cell.

-346-

52. A method for detecting types I or II interferons in a sample comprising:
- a) incubating the sample with the 5' regulatory element of the *mda-5* gene under conditions permitting binding of types I and II interferons transcriptional regulatory proteins to the regulatory elements; and
  - b) detecting the binding of the types I or II interferons transcriptional regulatory proteins to the regulatory elements, the binding indicating the presence of type I or type II interferons.
53. A method of claim 52, wherein the cell is a eukaryotic cell.
54. A method of claim 52, wherein the 5' regulatory element is linked to the native *mda-5* gene and the detection of binding is by the examination of the elevated expression of *mda-5* gene.
55. A method of claim 52, wherein the 5' regulatory element is linked to a marker gene.
56. A method of claim 55, wherein the marker gene is  $\beta$ -galactosidase, luciferase or CAT.
57. A method for identifying compound capable of inducing terminal differentiation in human melanoma cells comprising:
- a) incubating the human melanoma cells with an appropriate concentration of the compound; and

-347-

- b) detecting the expression of *mda-5* using the method of claim 50, the expression of *mda-5* gene indicating that the compound is capable of inducing terminal differentiation in human melanoma cells.
58. An isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-6*.
59. A cDNA of claim 58.
60. An isolated nucleic acid molecule of claim 58, wherein the protein is a human protein.
61. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of claim 58.
62. A method of detecting the expression of *mda-6* gene in a cell comprising:
- a) isolating the nucleic acids in the cell;
  - b) hybridizing the isolated nucleic acids with the nucleic acid molecules of claim 61 under conditions permitting hybrids formation; and
  - c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-6* gene in the cell.
63. A method for distinguishing a normal neuroectodermal cell from a malignant

-348-

neuroectodermal cell comprising detecting the expression of *mda-6* gene using the method of claim 62, the expression of *mda-6* gene indicating that the cell is normal neuroectodermal cell.

64. A method for monitoring response to topoisomerase inhibitor by a cell comprising detecting the expression of *mda-6* using the method of claim 62, the expression of *mda-6* indicating that the cell responds to the topoisomerase inhibitor.
65. A method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising:
  - a) incubating the human melanoma cells with an appropriate concentration of the compound; and
  - b) detecting the expression of *mda-6* using the method of claim 62, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human melanoma cells.
66. A method for identifying a compound capable of inducing terminal differentiation in human leukemia cells comprising:
  - a) incubating the human leukemia cells with an appropriate concentration of the compound; and
  - b) detecting the expression of *mda-6* using the method of claim 62, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human

-349-

leukemia cells.

67. A method for identifying a compound capable of inducing terminal differentiation in human lymphoma cells comprising:

- a) incubating the human lymphoma cells with an appropriate concentration of the compound; and
- b) detecting the expression of *mda-6* using the method of claim 62, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human lymphoma cells.

68. A method for identifying a compound capable of inducing terminal differentiation in human neuroblastoma cells comprising:

- a) incubating the human neuroblastoma cells with an appropriate concentration of the compound; and
- b) detecting the expression of *mda-6* using the method of claim 62, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human neuroblastoma cells.

69. A method for identifying compound capable of inducing terminal differentiation in human glioblastoma multiforme cells comprising:

- a) incubating the human glioblastoma cells with an appropriate concentration of the compound;

-350-

and

- b) detecting the expression of *mda-6* using the method of claim 62, the expression of *mda-6* gene indicating that the compound is capable of inducing terminal differentiation in human glioblastoma multiforme cells.

70. A method for distinguishing an early stage from a more progressed human melanoma cell comprising detecting the expression of *mda-6* gene in a cell using the method of claim 62, the expression of *mda-6* gene indicating that the cell is an early stage melanoma cell.

71. A method for reversing the malignant phenotype of cells comprising:

- (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element; and

- (b) introducing the linked *mda-6* gene into the malignant cells for the expression of the *mda-6* gene, thereby reversing the malignant phenotype of cells.

72. A method for reversing the malignant phenotype of cells comprising:

- (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element;

-351-

- (b) introducing the linked *mda-6* gene into the malignant cells; and
  - (c) placing the cells from step (b) in appropriate conditions to express the *mda-6* gene such that the expression of the *mda-6* gene will reverse the transforming phenotype of the malignant cells.
73. A method of reversing the phenotype of malignant cells in a subject comprising:
- (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element;
  - (b) introducing the linked *mda-6* gene into the malignant cells for the expression of the *mda-6* gene, thereby reversing the phenotype of the malignant cells.
74. A method of reversing the phenotype of malignant cells in a subject comprising:
- (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element;
  - (b) introducing the linked *mda-6* gene into the malignant cells of the subject; and
  - (c) inducing the expression of the *mda-6* gene which will reverse the transforming properties of the cells, thereby reversing the phenotype of the malignant cells in the subject.

-352-

75. A method of inducing growth suppression in tumorigenic and metastatic cells comprising:
- (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element;
  - (b) introducing the linked *mda-6* gene into the tumorigenic and metastatic cells; and
  - (c) inducing the expression of the *mda-6* gene, thereby inducing growth suppression in tumorigenic and metastatic cells.
76. A method of inducing terminal differentiation in tumorigenic and metastatic cells comprising:
- (a) linking the *mda-6* gene to a regulatory element such that the expression of the *mda-6* gene is under the control of the regulatory element;
  - (b) introducing the linked *mda-6* gene into the tumorigenic and metastatic cells; and
  - (c) inducing the expression of the *mda-6* gene, thereby inducing terminal differentiation in tumorigenic and metastatic cells.
77. A method of claim 71, 72, 73, 74, 75 or 76 wherein the cell is a melanoma cell.
78. A method of claim 71, 72, 73, 74, 75, or 76, wherein the cell is a leukemia cell.
79. A method of claim 71, 72, 73, 74, 75 or 76,



-353-

wherein the cell is a lymphoma cell.

80. A method of claim 71, 72, 73, 74, 75 or 76, wherein the cell is a neuroblastoma cell.
81. A method of claim 71, 72, 73, 74, 75 or 76, wherein the cell is a glioblastoma multiforme cell.
82. A method of claim 71, 72, 73, 74, 75 or 76, wherein the cell is a carcinoma cell.
83. A method of claim 71, 72, 73, 74, 75 or 76, wherein the regulatory element is a promoter.
84. A method of claim 83, wherein the promoter is a tissue-specific promoter.
85. A method of claim 83, wherein the promoter is an inducible promoter.
86. A method of claim 71, 72, 73, 74, 75 or 76, wherein the linked *mda-6* gene is introduced into the cells by naked DNA technology, retroviral vectors, antibody-coated liposomes, mechanical or electrical means.
87. A method of determining the stage of a melanoma comprising:
  - (a) obtaining appropriate amount of cells from the melanoma;
  - (b) measuring the expression level of the *mda-6* gene in the cells; and

-354-

(b) comparing the expression level with predetermined standards of normal and melanoma cells in different stages, thereby determining the stage of a melanoma.

88. A method of claim 87, wherein the expression is measured by the antibodies against the *mda-6* protein.
89. A method of claim 87, wherein the expression is measured by in situ hybridization.
90. A method for indicating the effectiveness of a treatment against cancer comprising measuring the expression level of *mda-6* gene in the cells of the cancer, the increase of the expression level indicating the effectiveness of the treatment.
91. A method of claim 90, wherein the cancer is melanoma
92. A method of claim 90, wherein the cancer is leukemia.
93. A method of claim 90, wherein the cancer is lymphoma.
94. A method of claim 90, wherein the cancer is neuroblastoma.
95. A method of claim 90, wherein the cancer is a glioblastoma multiforme tumor.
96. A method of claim 90, wherein the cancer is a carcinoma.

-355-

97. An isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-7*.
98. A cDNA of claim 97.
99. An isolated nucleic acid molecule of claim 98, wherein the protein is a human protein.
100. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of claim 97.
101. A method of detecting the expression of *mda-7* gene in a cell comprising:
  - a) isolating the nucleic acids in the cell;
  - b) hybridizing the isolated nucleic acids with the nucleic acid molecules of claim 97 under conditions permitting hybrids formation; and
  - c) detecting hybrids formed, the detection of the hybrids formed indicating the expression of *mda-7* gene in the cell.
102. A method for determining whether a cell is a melanoma cell or a carcinoma cell comprising detecting the expression of *mda-7* gene using the method of claim 101, the expression of *mda-7* gene indicating that the cell is a melanoma cell.
103. A method for identifying compound capable of inducing growth suppression in human melanoma cells comprising:

-356-

- a) incubating the human melanoma cells with an amount of the compound effective to induce growth suppression in human melanoma cells; and
- b) detecting the expression of *mda-7* using the method of claim 101, the expression of *mda-7* gene indicating that the compound is capable of inducing growth suppression in human melanoma cells.

104. A method for reversing the malignant phenotype of cells comprising:

- (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; and
- (b) introducing the linked *mda-7* gene into the malignant cells for the expression of the *mda-7* gene, thereby reversing the malignant phenotype of cells.

105. A method for reversing the malignant phenotype of cells comprising:

- (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element;
- (b) introducing the linked *mda-7* gene into the malignant cells; and
- (c) placing the cells from step (b) in appropriate conditions to express the *mda-7* gene such that

-357-

the expression of the *mda-7* gene will reverse the transforming phenotype of the malignant cells.

106. A method for reversing the phenotype of malignant cells in a subject comprising:

- (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element; and
- (b) introducing the linked *mda-7* gene into the malignant cells for the expression of the *mda-7* gene, thereby reversing the phenotype of the malignant cells.

107. A method to reversing the phenotype of malignant cells in a subject comprising:

- (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element;
- (b) introducing the linked *mda-7* gene into the malignant cells of the subject; and
- (c) inducing the expression of the *mda-7* gene which will reverse the transforming properties of the cells, thereby reversing the phenotype of the malignant cells in the subject.

108. A method of inducing growth suppression in tumorigenic and metastatic cells comprising:

-358-

- (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element;
  - (b) introducing the linked *mda-7* gene into the tumorigenic and metastatic cells; and
  - (c) inducing the expression of the *mda-7* gene, thereby inducing growth suppression in tumorigenic and metastatic cells.
109. A method of inducing terminal differentiation in tumorigenic and metastatic cells comprising:
- (a) linking the *mda-7* gene to a regulatory element such that the expression of the *mda-7* gene is under the control of the regulatory element;
  - (b) introducing the linked *mda-7* gene into the tumorigenic and metastatic cells; and
  - (c) inducing the expression of the *mda-7* gene, thereby inducing terminal differentiation in tumorigenic and metastatic cells.
110. A method of claim 104, 105, 106, 107, 108 or 109 wherein the cell is a melanoma cell.
111. A method of claim 104, 105, 106, 107, 108 or 109, wherein the cell is a leukemia cell.
112. A method of claim 104, 105, 106, 107, 108 or 109, wherein the cell is a lymphoma cell.
113. A method of claim 104, 105, 106, 107, 108 or 109,

-359-

wherein the cell is a neuroblastoma cell.

114. A method of claim 104, 105, 106, 107, 108 or 109, wherein the cell is a glioblastoma multiforme cell.

115. A method of claim 104, 105, 106, 107, 108 or 109, wherein the regulatory element is a promoter.

116. A method of claim 115, wherein the promoter is a tissue-specific promoter.

117. A method of claim 115, wherein the promoter is an inducible promoter.

118. A method of claim 104, 105, 106, 107, 108 or 109, wherein the linked *mda-7* gene is introduced into the cells by naked DNA technology, retroviral vectors, antibody-coated liposomes, mechanical or electrical means.

119. A method of determining the stage of a melanoma comprising:

(a) obtaining appropriate amount of cells from the melanoma;

(b) measuring the expression level of the *mda-7* gene in the cells; and

(b) comparing the expression level with predetermined standards of normal and melanoma cells in different stages, thereby determining the stage of a melanoma.

120. A method of claim 119, wherein the expression is measured by the antibodies against the *mda-7* protein.

-360-

121. A method of claim 119, wherein the expression is measured by in situ hybridization.
122. A method for indicating the effectiveness of a treatment against cancer comprising measuring the expression level of *mda-7* gene in the cells of the cancer, the increase of the expression level indicating the effectiveness of the treatment.
123. A method of claim 122, wherein the cancer is melanoma
124. A method of claim 122, wherein the cancer is leukemia.
125. A method of claim 122, wherein the cancer is lymphoma.
126. A method of claim 122, wherein the cancer is neuroblastoma.
127. A method of claim 122, wherein the cancer is a glioblastoma multiforme tumor.
128. A method of determining whether a cell is senescent comprising detecting the expression of *mda-7*, the expression of the *mda-7* gene indicating that the cell is senescent.
129. A method of identifying a compound inhibiting senescence comprising:
  - a) incubating a plurality of cells with an appropriate amount of a compound;



-361-

- b) detecting the expression of *mda-7*, the inhibition of the expression of *mda-7* indicating that the compound is inhibiting senescence.
130. An isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-8*.
131. A cDNA of claim 130.
132. A isolated nucleic acid molecule of claim 130, wherein the protein is a human protein.
133. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of claim 130.
134. A method of detecting the expression of *mda-8* gene in a cell comprising:
- a) isolating the nucleic acids in the cell;
  - b) hybridizing the isolated nucleic acids with the nucleic acid molecules of claim 132 under conditions permitting hybrids formation; and
  - c) detecting hybrids formed, detection of the hybrids indicating the expression of *mda-8* gene in the cell.
135. A method for distinguishing a normal glial cell from a malignant astrocytoma cell comprising detecting the expression of *mda-8* gene using the method of claim 134, the expression of *mda-8* gene

-362-

indicating that the cell is a normal glial cell.

136. A method for detecting type II interferons in a sample comprising:

a) incubating the sample with a target cell containing the 5' regulatory element of *mda-8* permitting binding of type II interferon transcriptional regulatory proteins to the 5' regulatory elements; and

b) detecting the binding, the binding indicating the presence of type II interferons in the sample.

137. A method of claim 136, wherein the cell is a eukaryotic cell.

138. A method of claim 136, wherein the 5' regulatory element is linked to the native *mda-8* gene and the detection of binding is by the examination of the elevated level of *mda-8* gene expression.

139. A method of claim 136, wherein the 5' regulatory element is linked to a marker gene.

140. A method of claim 139, wherein the marker gene is  $\beta$ -galactosidase or CAT.

141. A method for identifying a compound capable of inducing terminal differentiation in human melanoma cells comprising:

a) contacting the human melanoma cells with an appropriate concentration of the compound; and

-363-

- b) detecting the expression of *mda-8* gene using the method of claim 134, the expression of *mda-8* gene indicating that the compound is capable of inducing terminal differentiation in human melanoma cells.
142. An isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-9*.
143. A cDNA of claim 142.
144. An isolated nucleic acid molecule of claim 142, wherein the protein is a human protein.
145. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid molecule of claim 142.
146. A method of detecting the expression of *mda-9* gene in a cell comprising:
- a) isolating the nucleic acids in the cell;
  - b) hybridizing the isolated nucleic acids with the nucleic acid molecules of claim 145 under conditions permitting hybrids formation; and
  - c) detecting hybrids formed, detection of hybrids formed indicating the expression of *mda-9* gene in the cell.
147. A method for indicating the stage of progression of a human melanoma cell comprising detecting the expression of *mda-9* gene using the method of claim

-364-

146, the expression of *mda-9* gene indicating the stage of progression of a human melanoma cell.

148. A method for identifying compound capable of inducing terminal differentiation in human melanoma cells comprising:

- a) incubating appropriate concentration of the human melanoma cells with an appropriate concentration of the compound;
- b) detecting the expression of *mda-9* using the method of claim 146, the expression of *mda-9* gene indicating that the compound is capable of inducing terminal differentiation in human melanoma cells.

149. A method for identifying compound capable of inducing specific patterns of DNA damage caused by UV irradiation and gamma irradiation in human melanoma cells comprising:

- a) inducing appropriate concentration of the human melanoma cells with an appropriate concentration of the compound; and
- b) detecting the expression of *mda-9* using the method of claim 146, the expression of *mda-9* gene indicating that the compound is capable of inducing specific patterns of DNA damage caused by UV irradiation and gamma irradiation in human melanoma cells.

150. An isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation

-365-

associated gene designated *mda-11*.

151. A cDNA of claim 150.

152. An isolated nucleic acid molecule of claim 150, wherein the protein is a human protein.

153. An isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-14*.

154. A cDNA of claim 153.

155. An isolated nucleic acid molecule of claim 153, wherein the protein is a human protein.

156. An isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-17*.

157. A cDNA of claim 156.

158. An isolated nucleic acid molecule of claim 156, wherein the protein is a human protein.

159. An isolated nucleic acid molecule encoding a protein produced by a melanoma differentiation associated gene designated *mda-18*.

160. A cDNA of claim 159.

161. An isolated nucleic acid molecule of claim 159, wherein the protein is a human protein.

162. A method of detecting expression of a melanoma

-366-

differentiation associated gene in a cell which comprises obtaining total RNA from the cell, contacting the RNA so obtained with a labelled nucleic acid molecule of specific *mda* gene under hybridizing conditions, determining the presence of RNA hybridized to the molecule, and thereby detecting the expression of the melanoma differentiation associated gene in the cell.

163. A method of detecting expression of a melanoma differentiation associated gene in tissue sections which comprises contacting the tissue sections with a labelled nucleic acid molecule of specific *mda* gene under hybridizing conditions, determining the presence of RNA hybridized to the molecule, and thereby detecting the expression of the melanoma differentiation associated gene in tissue sections.
164. An isolated mammalian nucleic acid molecule of claim 27, 31, 46, 58, 97, 130, 142, 150, 153, 156 or 159 operatively linked to a promoter of RNA transcription.
165. A vector which comprises the isolated mammalian nucleic acid molecule of claims 27, 31, 46, 58, 97, 130, 142, 150, 153, 156 or 159.
166. A vector of claim 165, wherein the vector is a plasmid.
167. The plasmid of claim 166 designated *mda-1* (ATCC Accession No. 75582).
168. The plasmid of claim 166 designated *mda-4* (ATCC Accession No. 75583).

-367-

169. The plasmid of claim 166 designated *mda-5* (ATCC Accession No. 75584).
170. The plasmid of claim 166 designated *mda-6* (ATCC Accession No. 75585).
171. The plasmid of claim 166 designated *mda-6.3'* (ATCC Accession No. 75903).
172. The plasmid of claim 166 designated *mda-6.5'* (ATCC Accession No. 75904).
173. The plasmid of claim 166 designated *mda-7* (ATCC Accession No. 75586).
174. The plasmid of claim 166 designated *mda-7.3'* (ATCC Accession No. 75905).
175. The plasmid of claim 166 designated *mda-7.5'* (ATCC Accession No. 75906).
176. The plasmid of claim 166 designated *mda-8* (ATCC Accession No. 75587).
177. The plasmid of claim 166 designated *mda-9* (ATCC Accession No. 75588).
178. The plasmid of claim 166 designated *mda-11* (ATCC Accession No. 75589).
179. The plasmid of claim 166 designated *mda-14* (ATCC Accession No. 75590).
180. The plasmid of claim 166 designated *mda-17* (ATCC Accession No. 75591).

-368-

181. The plasmid of claim 166 designated *mda-18* (ATCC Accession No. 75592).
182. A host vector system for the production of a polypeptide having the biological activity of a protein encoded by melanoma differentiation associated gene which comprises the vector of claim 110 and a suitable host.
183. A host vector system of claim 182, wherein the suitable host is a bacterial cell, insect cell, or mammalian cell.
184. A method of producing a polypeptide having the biological activity of a protein encoded by melanoma differentiation associated gene which comprises growing the host cells of the host vector system of claim 123 under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.
185. A mammalian cell comprising the vector of claim 165.
186. Purified protein encoded by the isolated nucleic acid molecule of claim 27, 31, 46, 58, 97, 130, 142, 150, 153, 156 or 159.
187. A method to produce antibody using the purified protein or portion thereof of claim 187.
188. A method of claim 187, wherein the antibody is monoclonal.
189. An antibody capable of binding to the purified



-369-

protein of claim 186.

190. A method for distinguishing a fibroblast or epithelial cell from a melanoma or central nervous system cell using the method of claim 35, the expression of *mda-4* indicating that the cell is a melanoma cell or a central nervous system lineage cell.
191. A method for distinguishing an adenocarcinoma cell from a carcinoma cell comprising detecting the expression of *mda-6* gene using the method of claim 62, the expression of *mda-6* gene indicating that the cell is a carcinoma cell.
192. A method for monitoring the response of a cell to an anticancer agent such as actinomycin-D or adriamycin comprising detecting the expression of *mda-6* gene using the method of claim 62, the expression of *mda-6* gene indicating that the cell responds to the anticancer agent.
193. A method for distinguishing a melanocyte or early stage melanoma cell from an advanced metastatic melanoma cell using the method of claim 101, the expression of *mda-7* gene indicating that the cell is a melanocyte or early stage melanoma cell.
194. A method for distinguishing a fibroblast from an epithelial cell using the method of claim 101, the expression of *mda-7* gene indicating that the cell is a fibroblast.
195. A method for monitoring the response of a cell to an anticancer agent such as adriamycin or

-370-

vincristine comprising detecting the expression of *mda-7* gene using the method of claim 101, the expression of *mda-7* gene indicating that the cell responds to the anticancer agent.

196. A method for monitoring the response of a cell to DNA damage induced by UV irradiation comprising detecting the expression of *mda-7* gene using the method of claim 101, the expression of *mda-7* gene indicating that the cell responds to the DNA damage.
197. A method for monitoring the response of a cell to an anticancer agent such as actinomycin D, adriamycin or cis-platinum comprising detecting the expression of *mda-8* gene using the method of claim 134, the expression of *mda-8* gene indicating that the cell responds to the anticancer agent.
198. A method for monitoring the response of a cell to DNA damage induced by UV irradiation comprising detecting the expression of *mda-8* using the method of claim 134, the expression of *mda-8* gene indicating that the cell responds to the DNA damage.
199. A method for identifying the presence of tumor necrosis factor or a similarly acting agent comprising detecting the expression of *mda-9* gene using the method of claim 146, the expression of *mda-9* gene indicating that the tumor necrosis factor or similar agent is present.
200. A method for monitoring the response of a cell to an anticancer agent such as phenyl butyrate or VP-

-371-

16 comprising detecting the expression of *mda-9* gene using the method of claim 146, the expression of *mda-9* gene indicating that the cell responds to the anticancer agent.

201. A method of claim 187, wherein the antibody is polyclonal.

1/45

FIGURE 1A

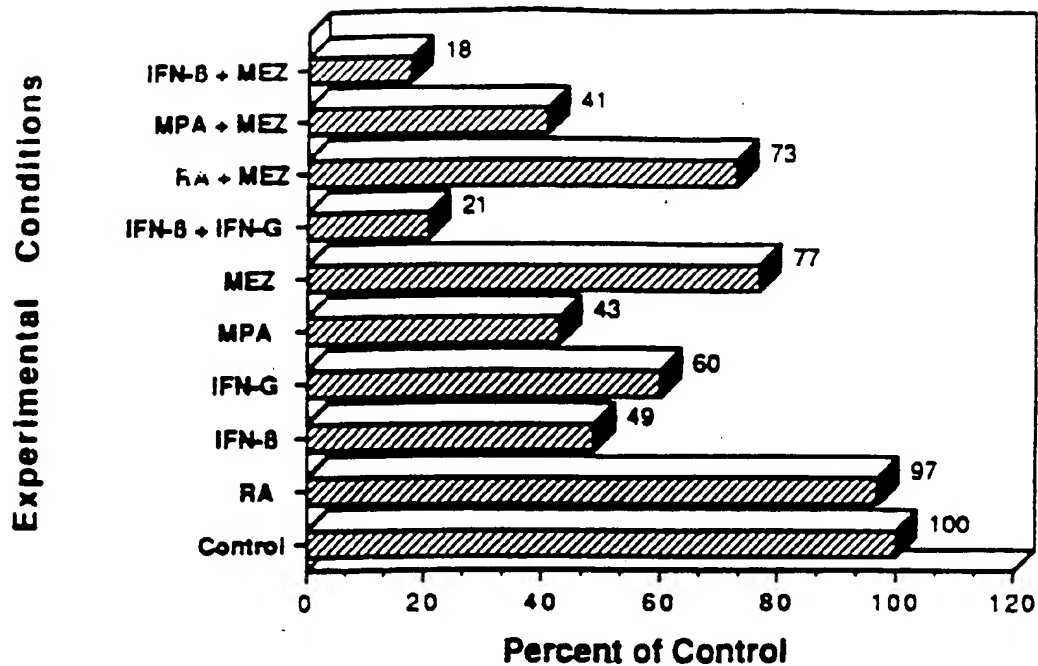
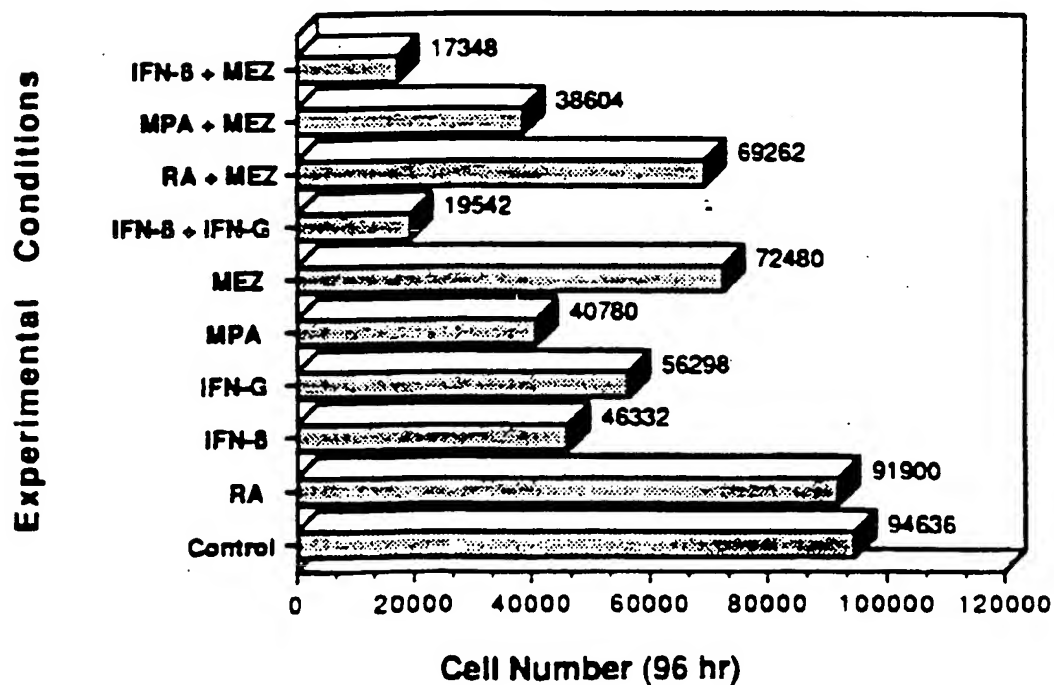
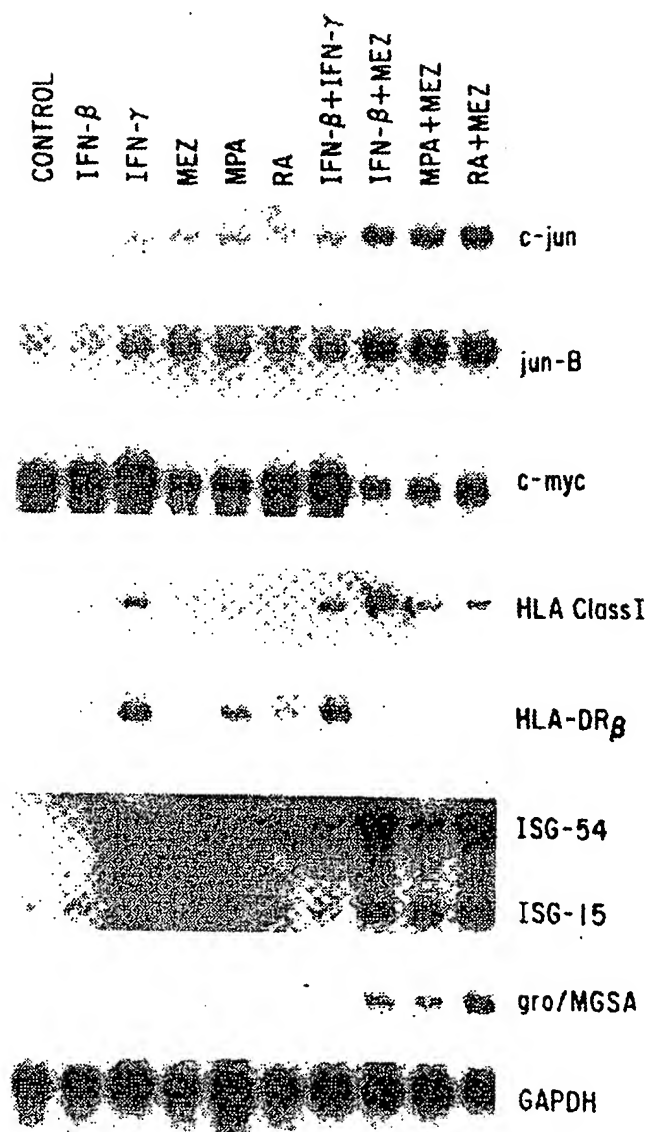


FIGURE 1B



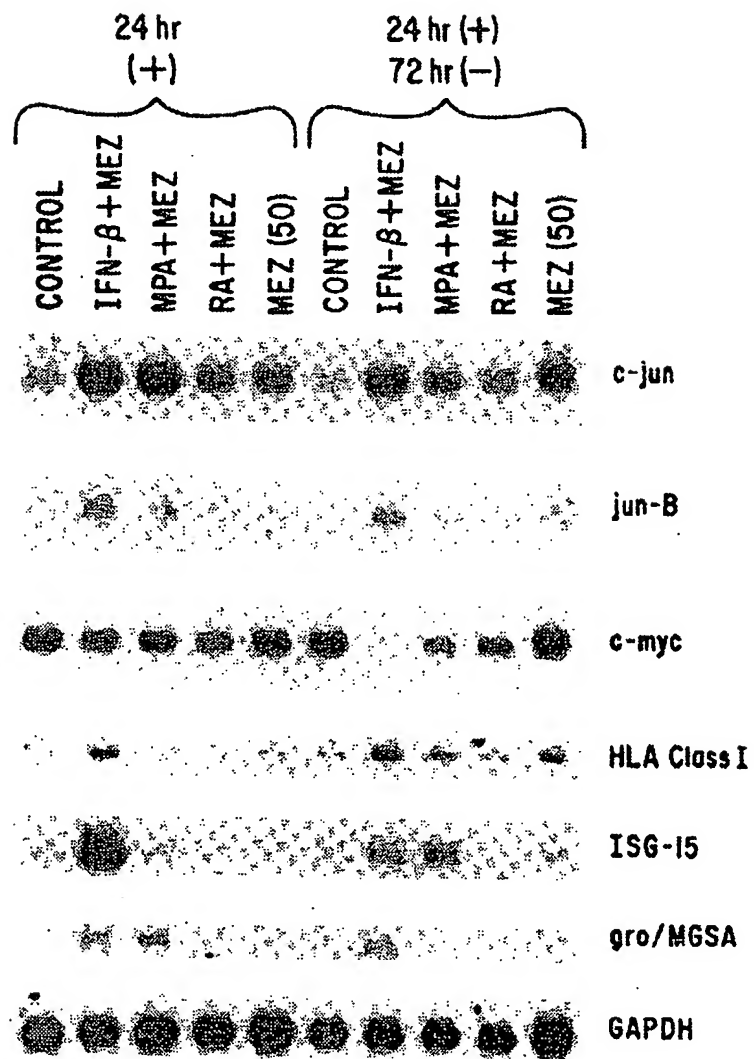
2/45

FIGURE 2



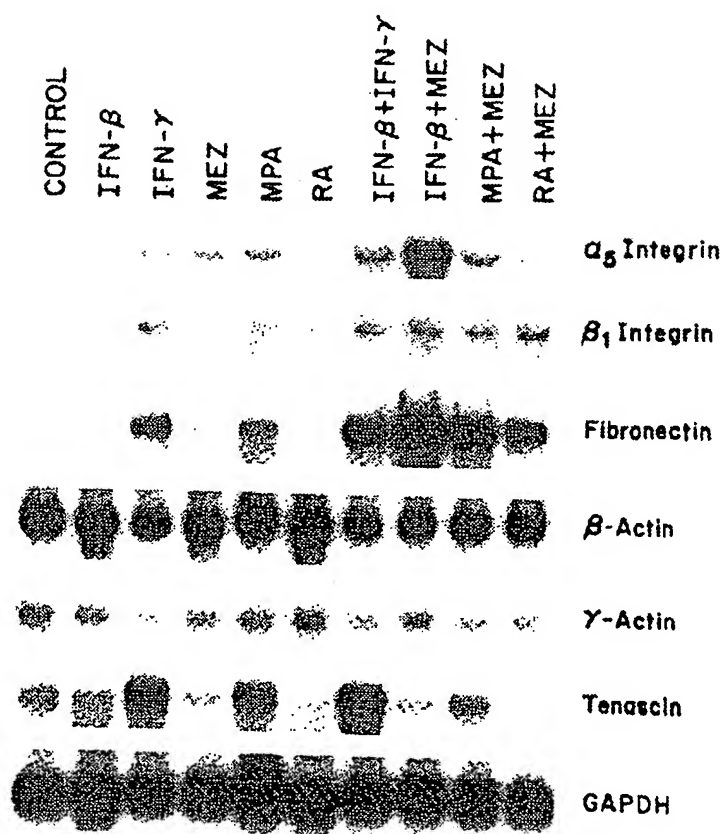
3/45

FIGURE 3



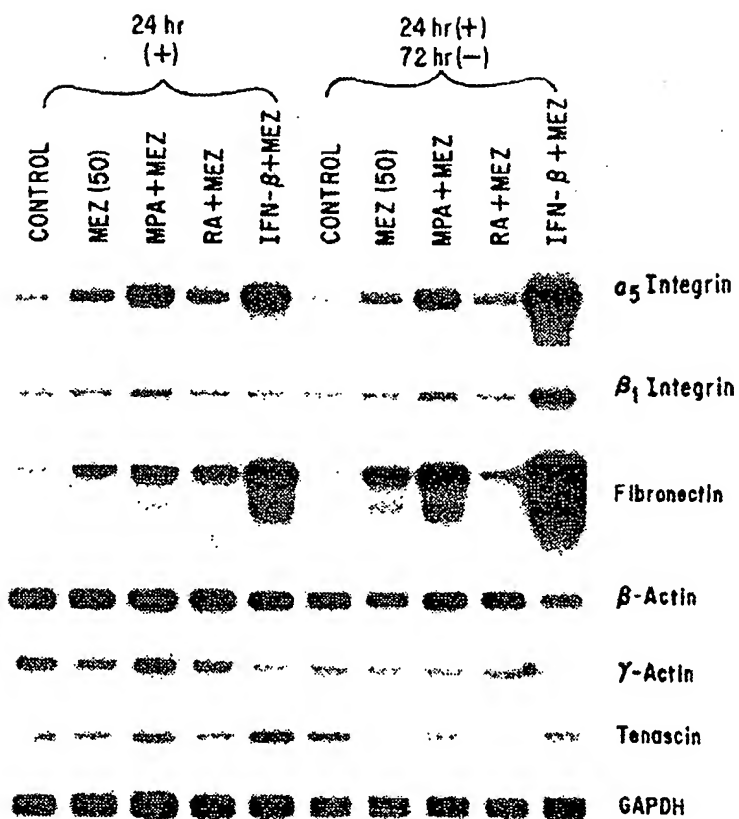
4/45

FIGURE 4



5/45

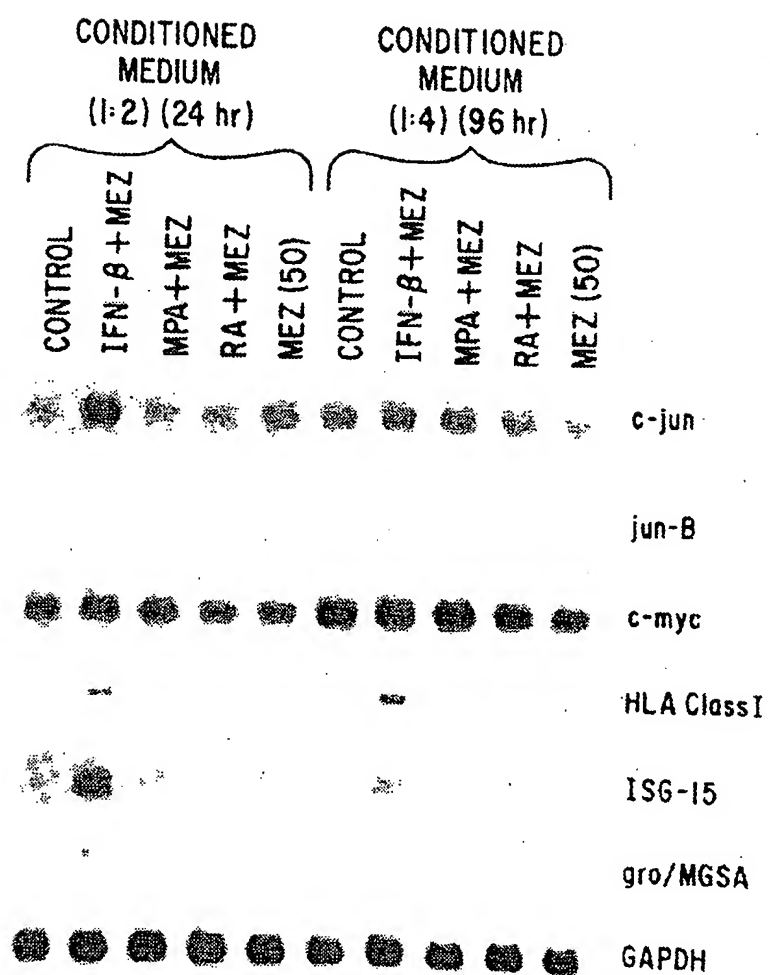
FIGURE 5





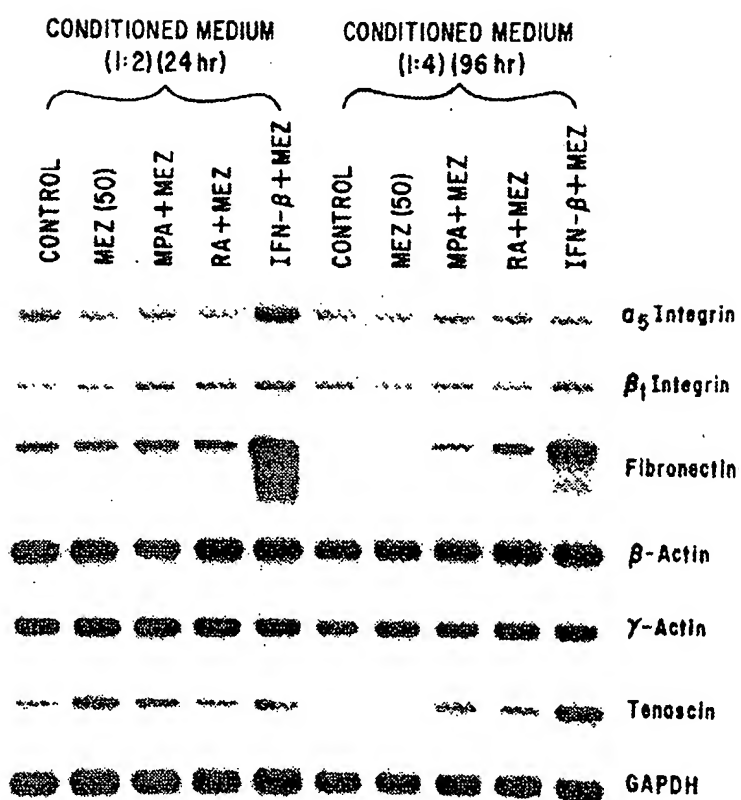
6/45

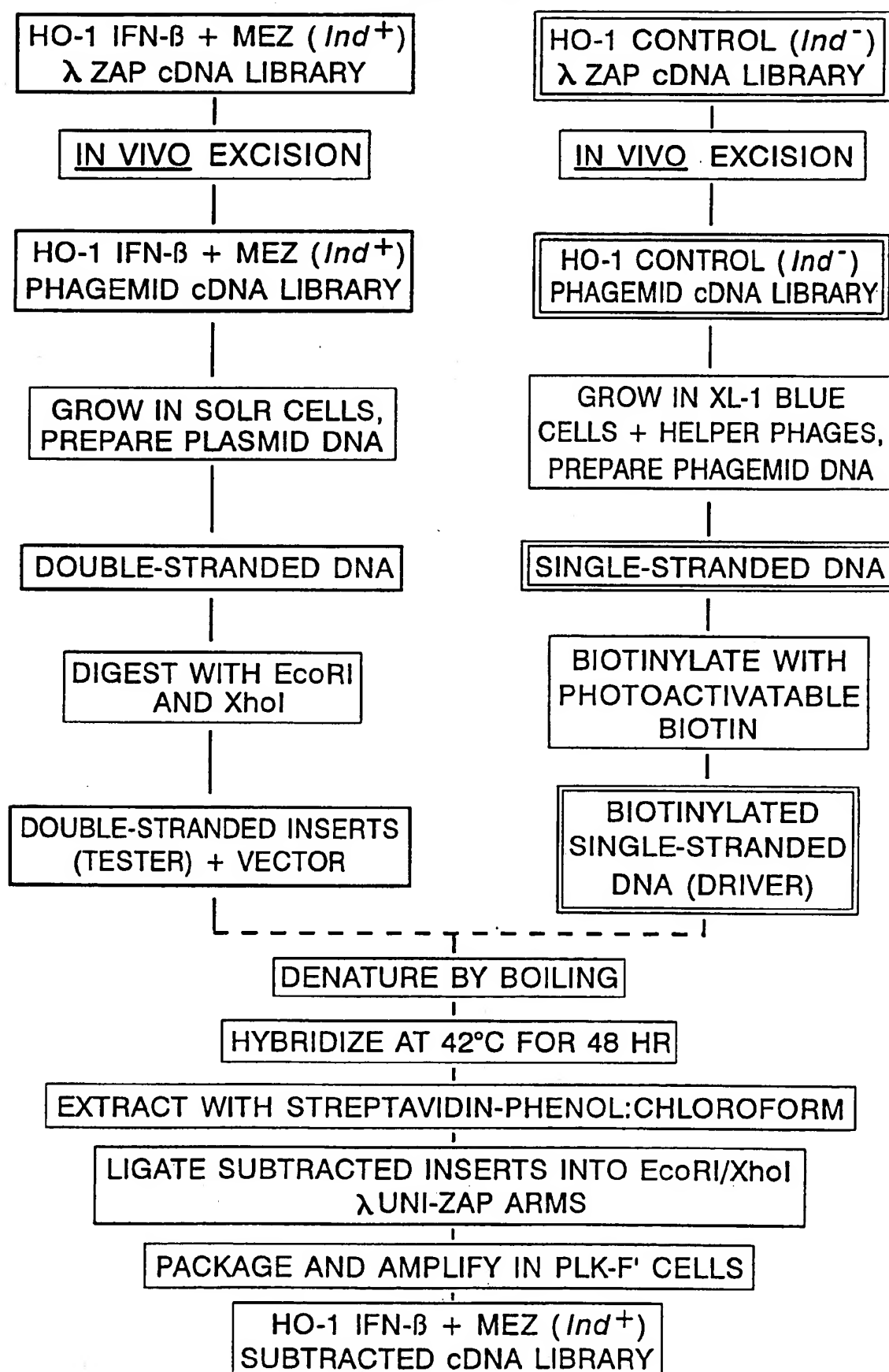
FIGURE 6



7/45

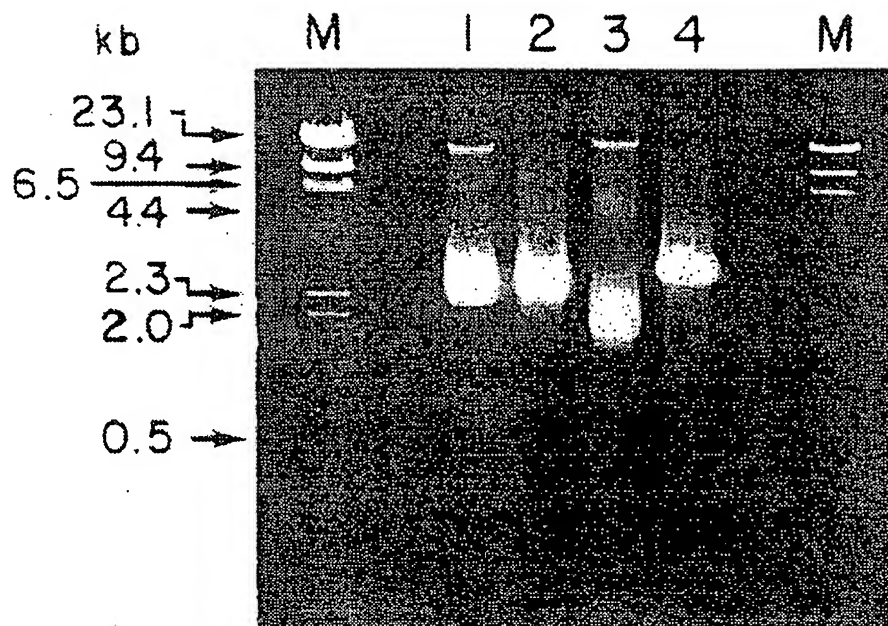
FIGURE 7



8/45  
FIGURE 8

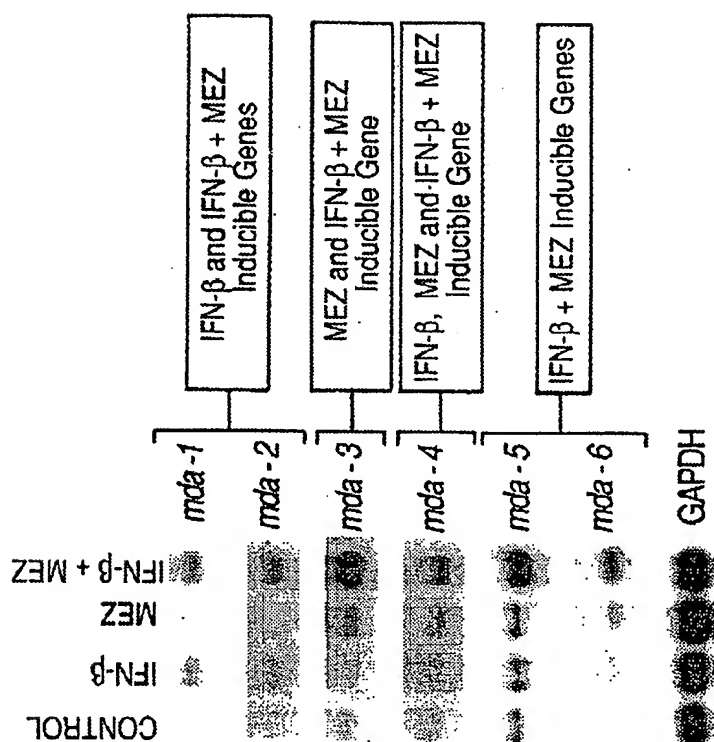
9/45

FIGURE 9



10/45

FIGURE 10



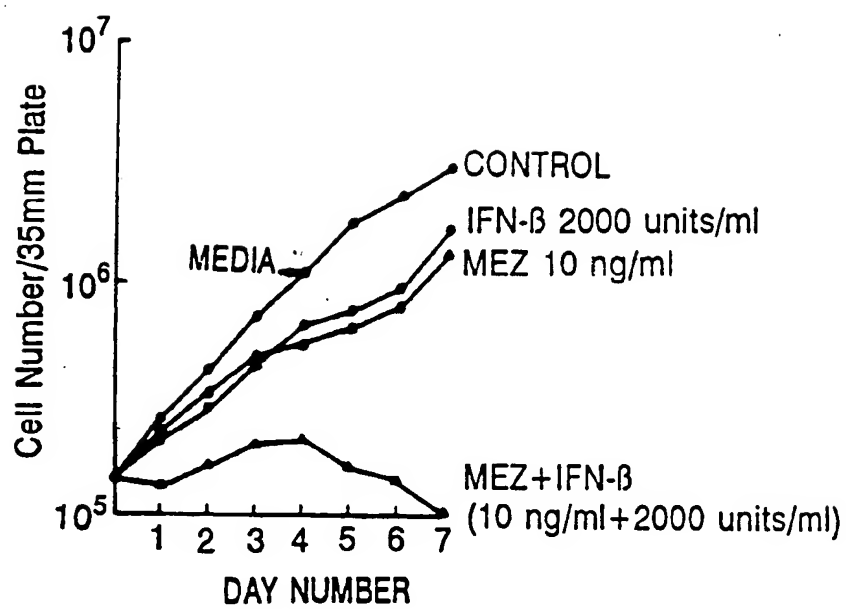
11/45

FIGURE 11

1 TCC TCC TCT GCA CCA TGG C TCT C TCA ACC AGT TCT CTG CAT CACT TGG C T 50  
|||||  
112 TCC TCC TCT GCA CCA TGG C TCT C TCA ACC AGT TCT CTG CAT CACT TGG C T 161  
51 GCT GAC AC GCG CCG ACC GCG CTG CTT CAG CTAC ACC TCC CGC AGA TTCC 100  
|||||  
162 GCT GAC AC GCG CCG ACC GCG CTG CTT CAG CTAC ACC TCC CGC AGA TTCC 211  
101 ACA GGA ATT TCA TAG CTG ACT ACT TTG AGA CCG AGC AGC CAG TGG CTCC AAG C 150  
|||||  
212 ACA GGA ATT TCA TAG CTG ACT ACT TTG AGA CCG AGC AGC CAG TGG CTCC AAG C 261  
151 CCG GTG TCAT CTT CCT AAC CCG ACG ACC GCG CAG GGT CTG TGG TGA CCCC 200  
|||||  
262 CCG GTG TCAT CTT CCT AAC CCG ACG ACC GCG CAG GGT CTG TGG TGA CCCC 311  
201 AGT GAG GAG TGG GTCC AGA AAT ATG TCAG CCG ACC TGG AGC TGA GT 245  
|||||  
312 AGT GAG GAG TGG GTCC AGA AAT ATG TCAG CCG ACC TGG AGC TGA GT 356

12/45

FIGURE 12

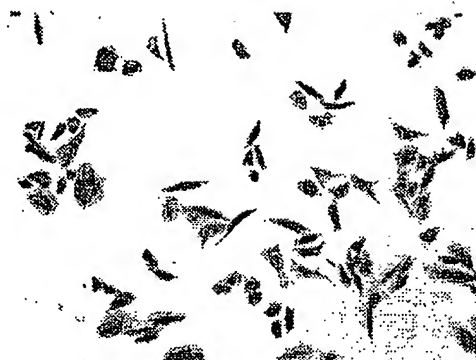


13/45

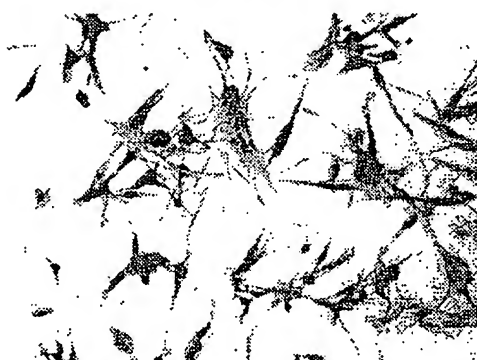
FIGURE 13



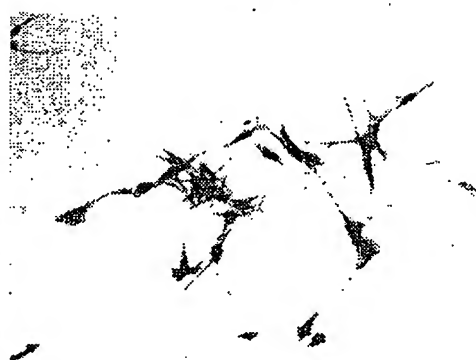
Control



IFN- $\beta$



MEZ



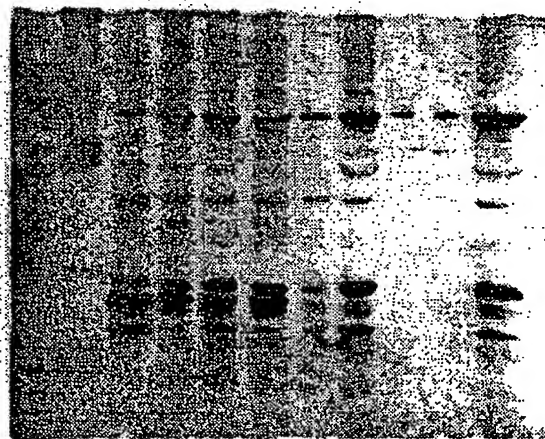
IFN- $\beta$  + MEZ



14/45

FIGURE 14

MARKERS  
 3T3T  
 FM 517 CONTROL  
 FM 517 B1000 + MEZ10  
 FM 517 B1000 + MEZ 50  
 FM 517 MEZ10 + MPA 3  
 FO-1 CONTROL  
 FO-1 B250 + MEZ50  
 FO-1 MEZ10 + MPA 3  
 HO-1 CONTROL

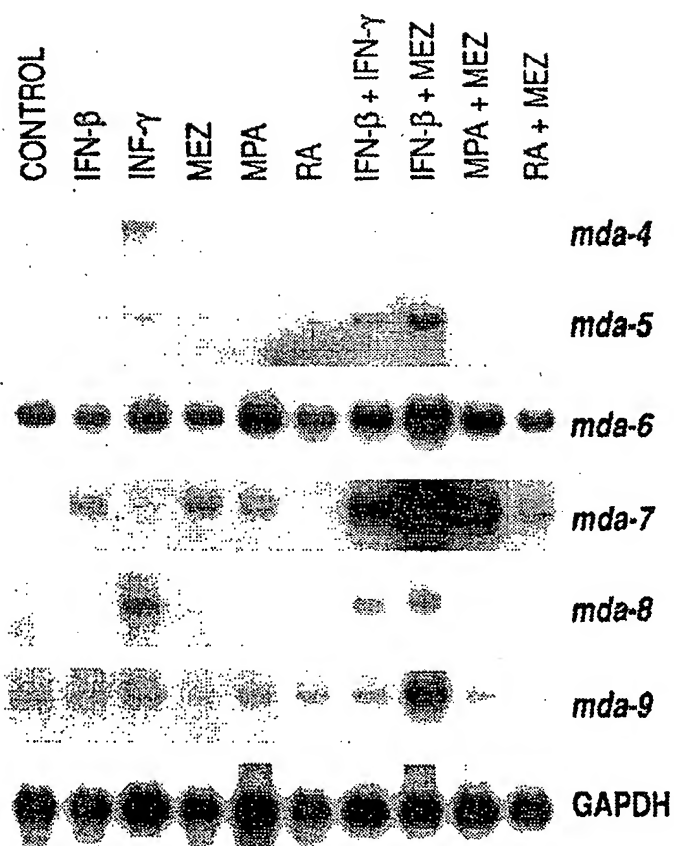


← hsp90

] P2Ps

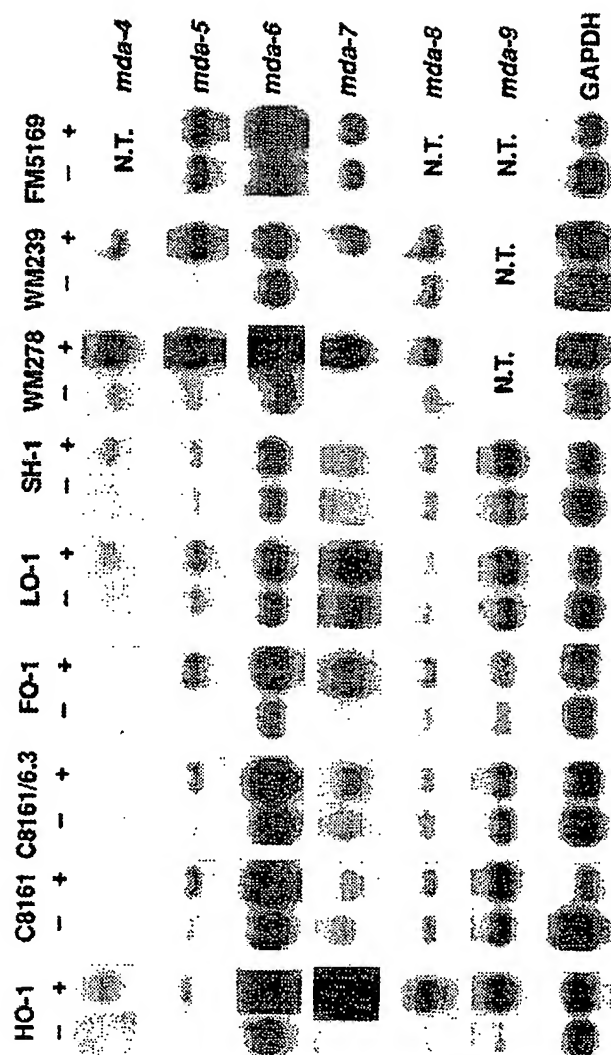
15/45

FIGURE 15



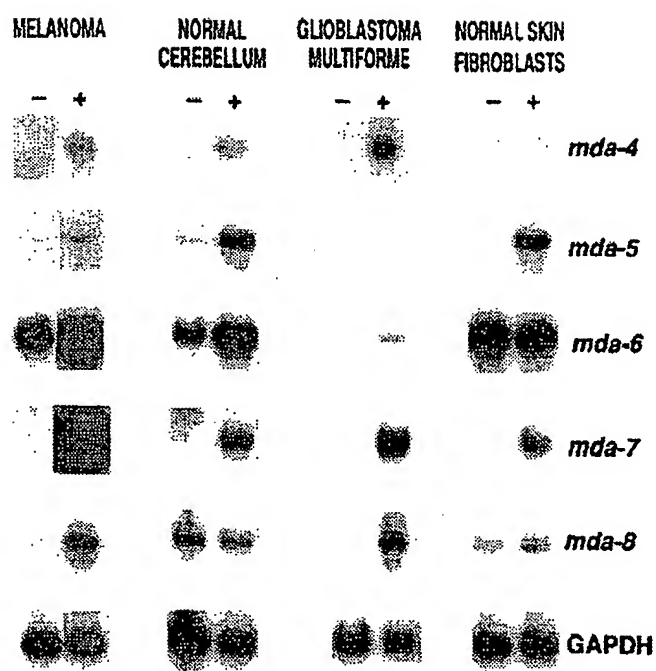
16/45

FIGURE 16



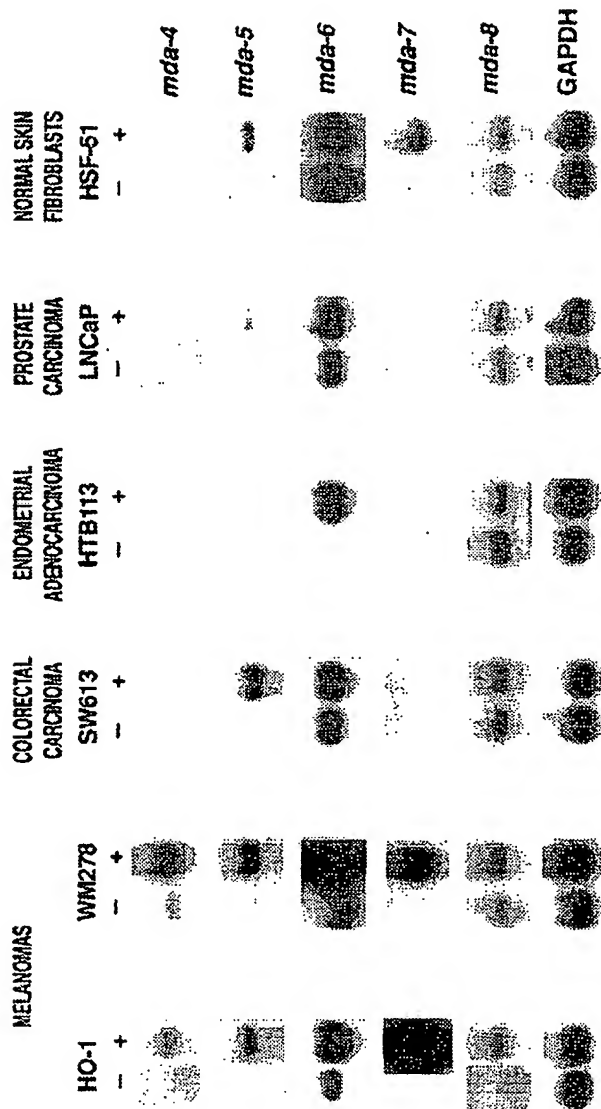
17/45

FIGURE 17



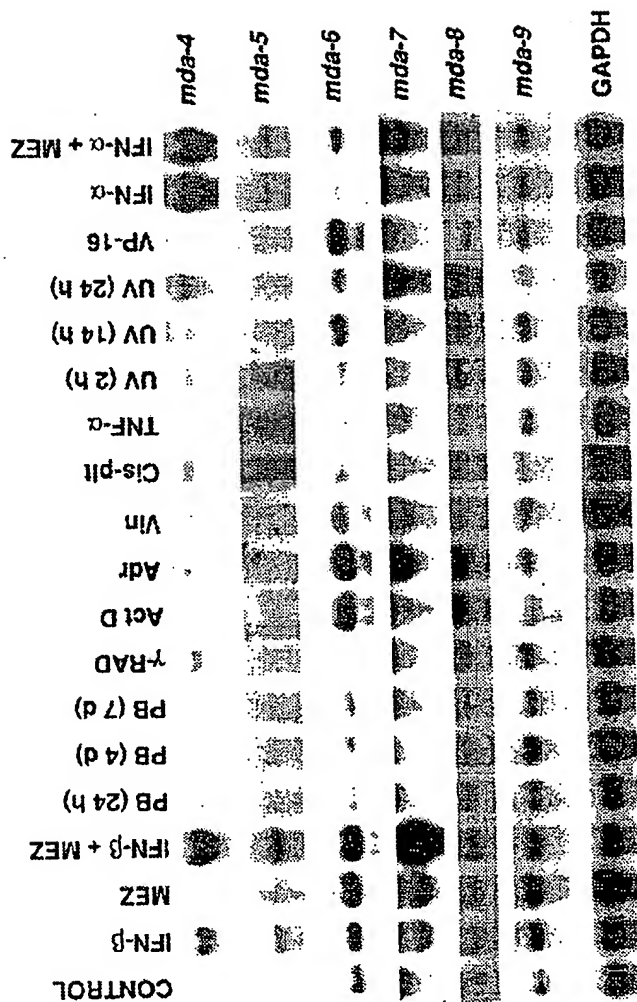
18/45

FIGURE 18



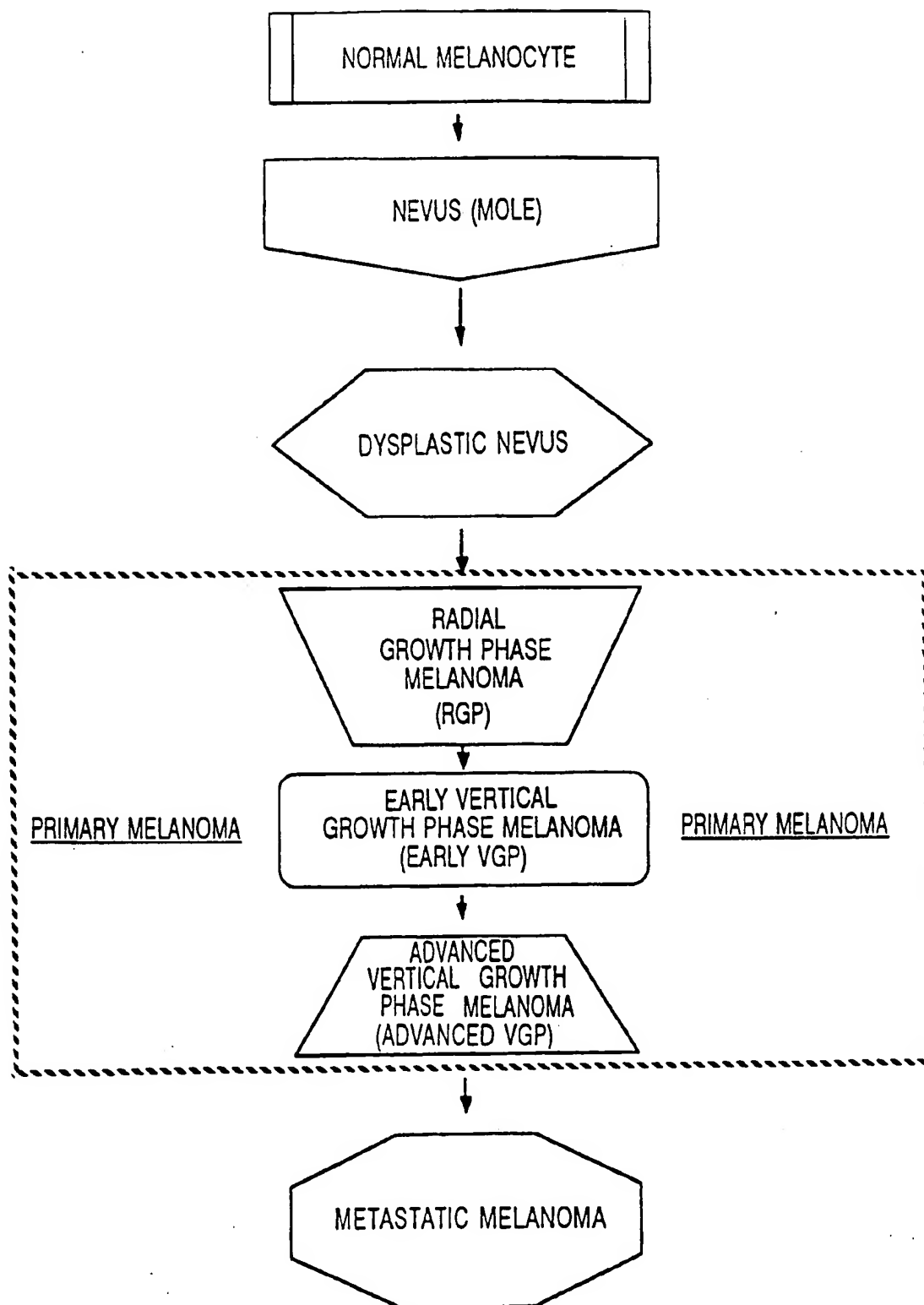
19/45

FIGURE 19



20/45

FIGURE 20



21/45

FIGURE 21A FIGURE 21B FIGURE 21C FIGURE 21D



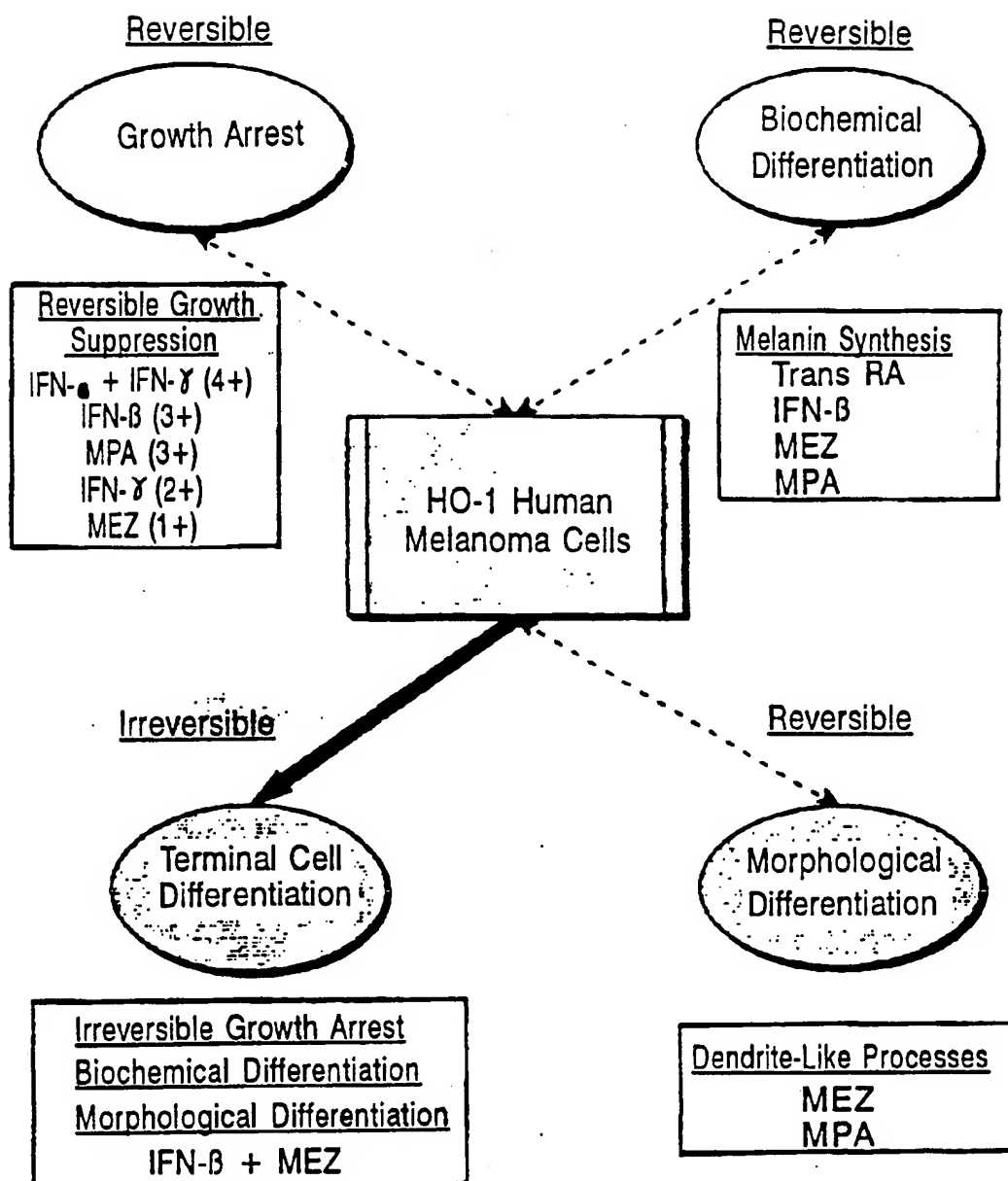
FIGURE 21E FIGURE 21F FIGURE 21G FIGURE 21H





22/45

FIGURE 22



23/45

FIGURE 23A

FIGURE 23A
FIGURE 23B

AGCTGAGGTGTGACAGCTGCCGAAGTCAGTTCCTTGTGGAGCCGGAGCTGGGCGGGAT 60  
TCGCCGAGGCAACGAGCACTCAGAGGAGCGGCCATGTCAGAAACCGGCTGGGGATGTCCG 120  
M S E P A G D V R  
TCAGAACCCCATGCGGCAGCAAGCCCTGCCCGCCCTCTTCGGCCCCAGTGGACAGCGAGCA 180  
Q N P C G S K A C R R L F G P V D S E Q  
GCTGAGCCGCGACTGTGATGCGCTAATGCGGGCTGCATCCAGGAGGCCCGTGAGCGATG 240  
L S R D C D A L M A G C I Q E A R E R W  
GAACTTCGACTTTGTACCCGAGACACCACCTGGAGGJTGACTTCGCCCTGGGAGCGTGTGCG 300  
N F D F V T E T P L E G D F A W E R V R  
GGCCCTTGGCCCTGCCCAAGCTCTACCTTCCCACGGGGCCCCGGAGCGCGGATGAGTT 360  
G L G L P K L Y L P T G P R R G R D E L  
GGGAGGAGGCAAGCGCCCTGGCACCTCACCTGCTCTGCTGCAGGGACAGCAGGAAGA 420  
G G R R P G T S P A L L Q G T A E E D  
CCATGTGGACCTGTCACTGTCTTGTACCCCTTGTGCCCTCGCTCAGGGAGCAGGCTGAAG 480  
H V D L S L S C T L V P R S G E Q A E G  
GTCCCCAGGTGGACCTGGAGACTCTCAGGGTCGAAACGGCGGCAGACCAGCATGACAGA 540  
S P G G P G D S Q G R K R Q T S M T D  
TTTCTACCACTCCAACGCGGCTGATCTTCTCCAAGAGGAAGCCCTAATCCGCCCCACAG 600  
F Y H S K R R L I F S K R K P \*  
GAAGCCTGCAGTCCCTGGAAGCGGAGGGCCCTCAAGGCCCGCTCTACATCTTCTGCCTTA 660  
GTCTCAGTTTGTGTCTTAATTATTATTGTTTAAATTAAACACCTCCTCATGTAC 720  
ATACCCCTGGCCCCCCTGCCCCCAGCCCTCTGGCATTAGAATTATTAAACAAAACATA 780

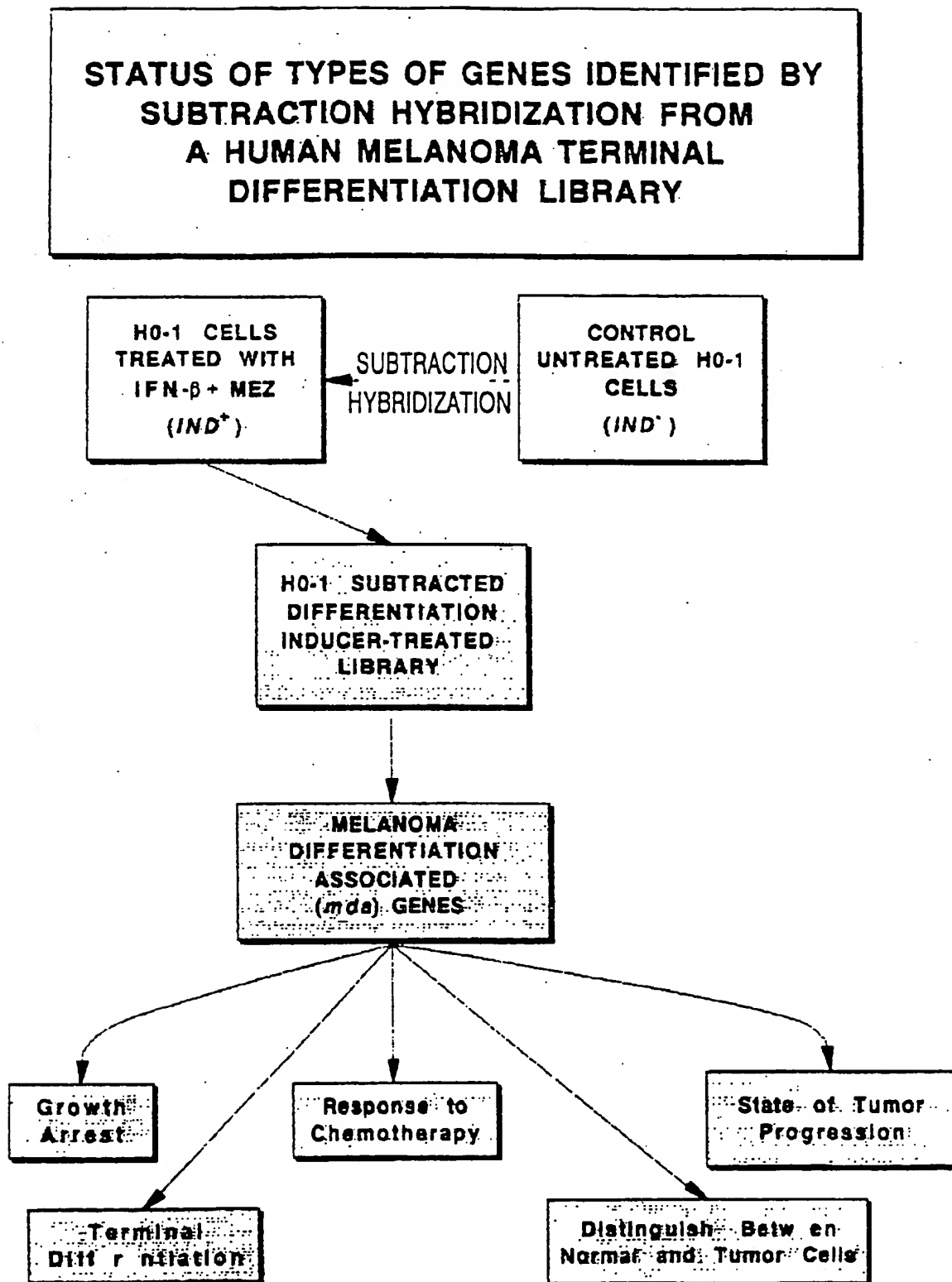
24/45

FIGURE 23B

GGCGGTTGAATGAGAGGTTCCCTAAGAGTGCTGGGCATTTTATTTATGAAATACTATTT 840  
AAAGCCCTCCCTCATCCCGTGTTCCTCTCTCCCGGAGGTTGGGTGGCCGGCTT 900  
CATGCCAGCTACTTCCTCCCTCCCACTTGTCCGCTGGGTACCCCTCTGGAGGGGTG 960  
GCTCCTTCCCATCGCTGTCACAGGCGGTTATGAAATTACCCCTTTCTCTGGACACTCAG 1020  
ACCTGAATTCTTTTCAATTGAGAAAGTAACAGATGGCACTTTGAAGGGGCCCTCACCGAG 1080  
TGGGGCATCATCAAAACTTTGGAGTCCCCCTCACCTCCTCTAAGGTTGGCAGGTGAC 1140  
CCTGAAGTGAGCACAGCCTAGGGCTAGCTGGGACCTGGTACCTCCTCTCTTGATA 1200  
CCCCCTCTGTCTTGTGAAGCAGGGGAAGTGGGTCCTGGAGCAGACACCCCGCCT 1260  
GCCCTCATGGCCCCCTGTGACCTGCACTGGGAGCCCGTCTCAGTGTGAGCCTTTTCCCT 1320  
CTTTGGCTCCCCGTACCTTTTGAAGAGCCCCAGCTACCTTCTCTCCAGCTGGGCTCT 1380  
GCAATTCCCCCTCTGCTGTCTCCCTCCCTTGTCTCTTTCCCTTCAGTACCCCTCTCAGCT 1440  
CCAGTGGCTCTGAGGTGCTGTCCCACTCCCTAGTTCTACCTCAGGCAGCTCAAGCAGCGCCG 1500  
AGGGACACACAAGAAGAGGCACTTCTAGTTCTACCTCAGGCAGCTCAAGCAGCGACCGC 1560  
CCCCCTCCTAGCTGTGGGGTGAAGGTCCCTATGTGGTGACAGGCCCTTTGAGTGGG 1620  
GTTATCTCTGTGTTAGGGGTATATGATGGGGGAGTAGATCTTCTAGGAGGAGACACTG 1680  
GCCCCCTCAATCGTCCAGCGACCTTCTCTATCCACCTCCCTCCCTCCAGTTCTATTGCAC 1740  
TTTGATTAGCAGCGGAACAAGGAGTCAGACATTTTAAGATGGTGCGCAGTAGAGGCTATGG 1800  
ACAGGGCATGCCACGTGGGCTCATATGGGGCTGGGAGTAGTGTCTTCTCTGGCACTAAC 1860  
GTTGAGCCCCCTGGAGGCACTGAAGTGTCTTAGTGTACTTGGAGTATTGGGGTCTGACCCCA 1920  
AACACCTTCCAGCTCCCTGTAACTACTGGCCCTGGACTGTTTCTCTCGGCTCCCCATGTG 1980  
TCCTGGTTCCCGTTTCTCCACCTAGACTGTAAACCTCTCGAGGGCAGGACACACCCCTG 2040  
TACTGTCTGTGCTTTTTCACAGCTCTCCACAAATGCTGAATAAACAGCAGGTGCTCAAT 2100  
AAATGATTCTTAGTGACTTTAAAAAATAAAAAAATAAAAAAATAAAAAA 2147

25/45

FIGURE 24



26/45

FIGURE 25A

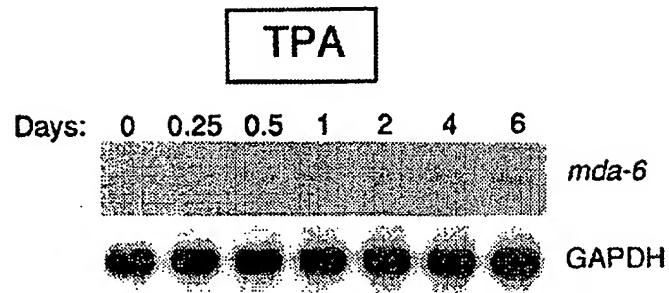
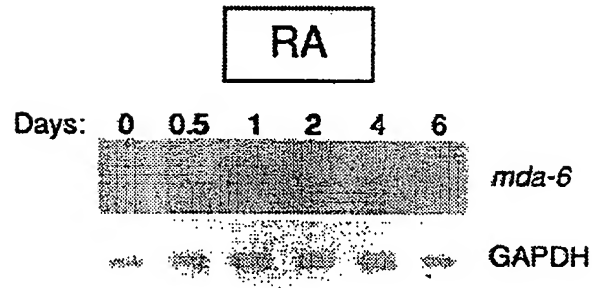


FIGURE 25B



27/45

FIGURE 26A

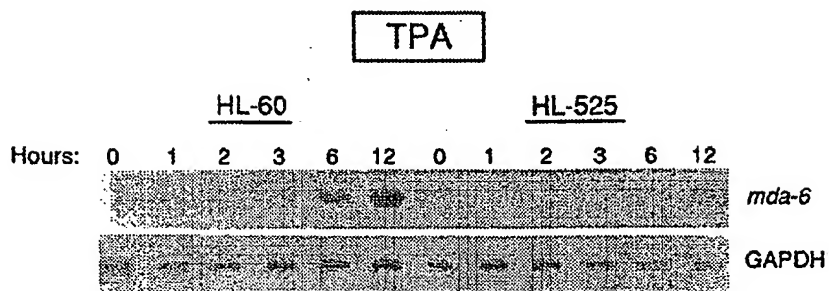


FIGURE 26B

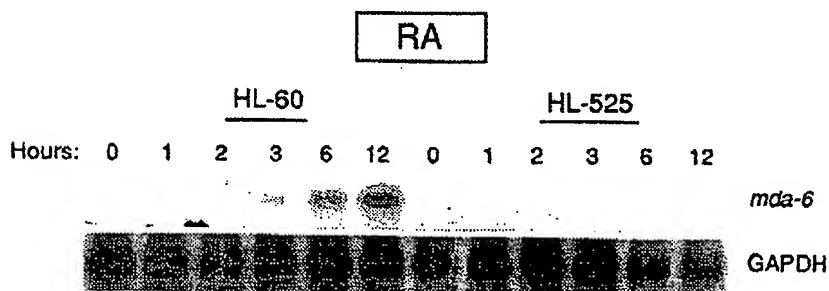
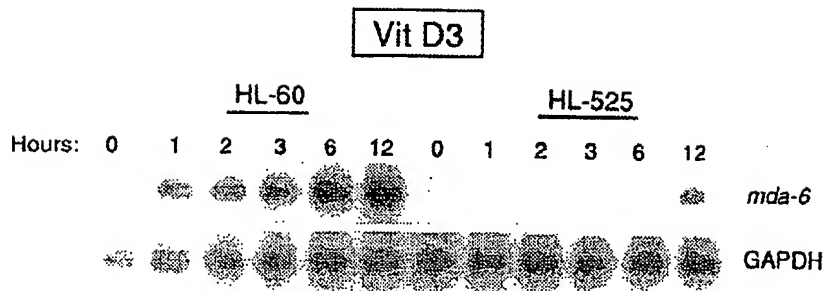


FIGURE 26C



28/45

FIGURE 27A

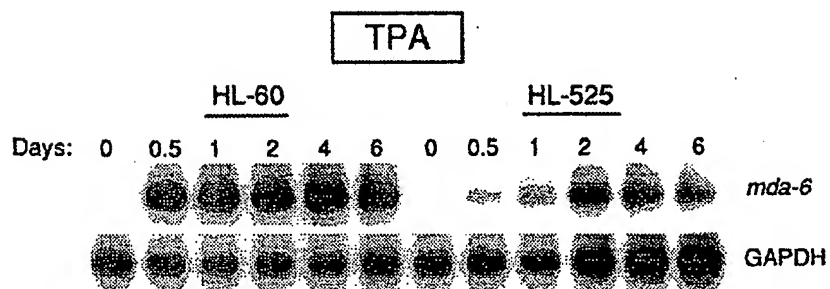


FIGURE 27B

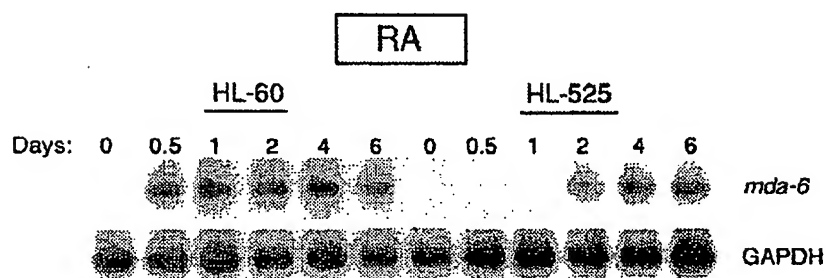
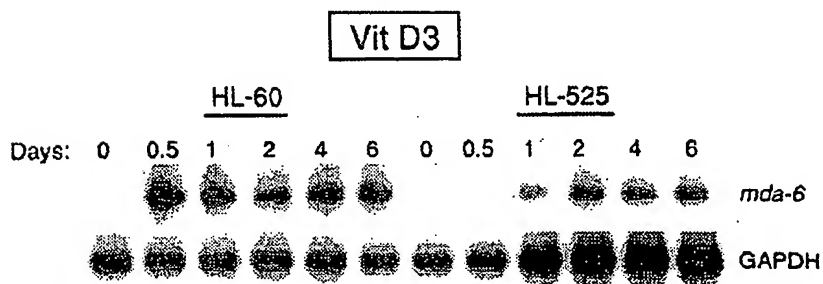
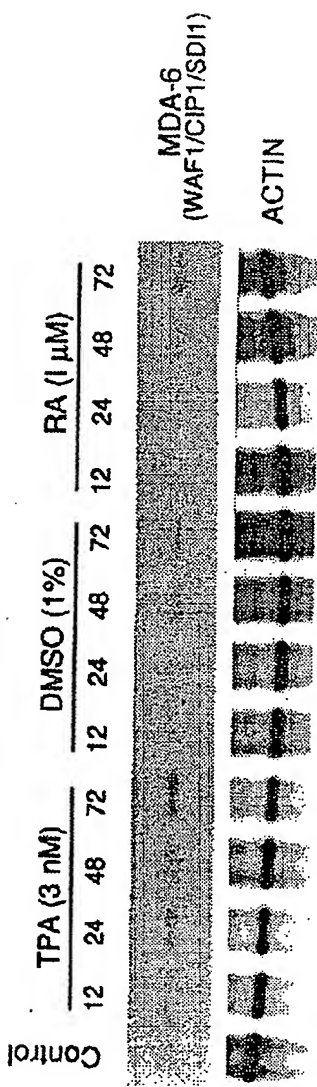


FIGURE 27C



29/45

FIGURE 28





30/45

FIGURE 29A

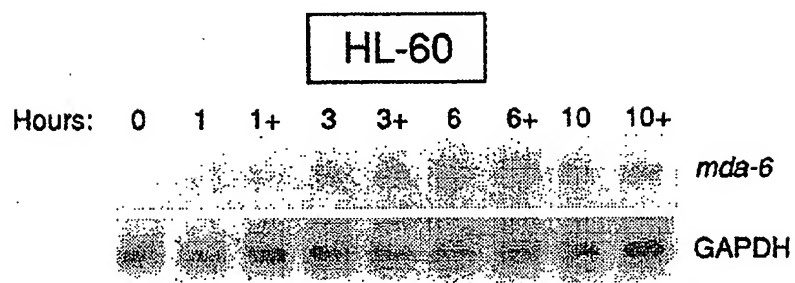
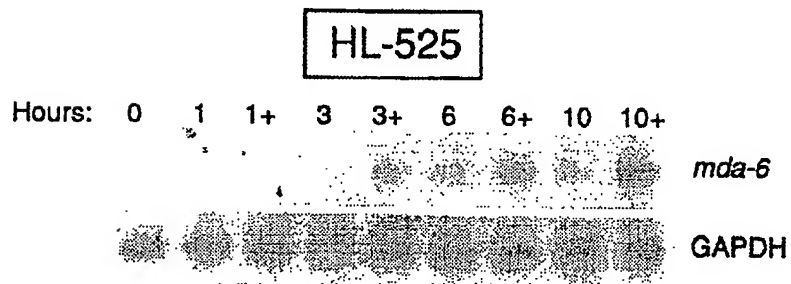


FIGURE 29B



31/45

FIGURE 30

ATGTCAGAACCGGCTGGGGATGTCCGTCAAGAACCCATGCGGCAGCAAGCCTGCCCGCGC  
M S E P A G D V R Q N P C G S K A C R R  
CTCTTCGGCCAGTGGACAGCAGCAGCTGAGCCGCGACTGTGATGCGCTAATGGCGGGC  
L F G P V D S E Q L S R D C D A L M A G  
TGCATCCAGGAGGCCCGTGAGCGATGGAACCTTCGACTTTGTCAACGAGACCACTGGAG  
C I Q E A R E R W N F D F V T E T P L E  
GGTGACTTCGCCCTGGGAGCGTGTGCGGGGCCCTTGGCCTGCCCAAGCTCTACCTTCCCACG  
G D F A W E R V R G L G L P K L Y L P T  
GGCCCCGGGAGCGGATGAGTTGGGAGGAGGCGGCGCCTGGCACCTCACCTGCT  
G P R R G R D E L G G G R R P G T S P A  
CTGCTGCAGGGACAGCAGAGGAAGACCATGTGGACCTGTCACTGTCTTGTACCTTGTG  
L L Q G T A E E D H V D L S L S C T L V  
CCTCGCTCAGGGAGCAGGCTGAAGGTCCTCCAGGTGGACCTGGAGACTCTCAGGGTCGA  
P R S G E Q A E G S P G G P G D S Q G R  
AAACGGCGCAGACCAGCATGACAGATTCTACCACTCCAACCGCGGTGATCTTCTCC  
K R R Q T S M T D F Y H S K R R L I F S  
AAGAGGAAGCCCTAA  
K R K P \*

32/45

FIGURE 31A    FIGURE 31B    FIGURE 31C

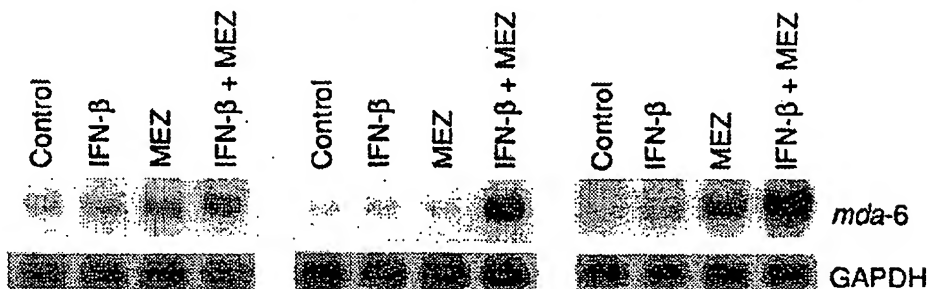


FIGURE 31D

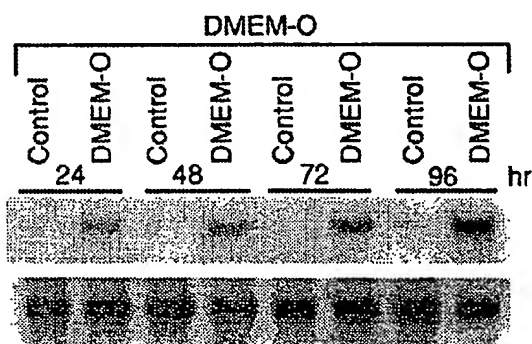
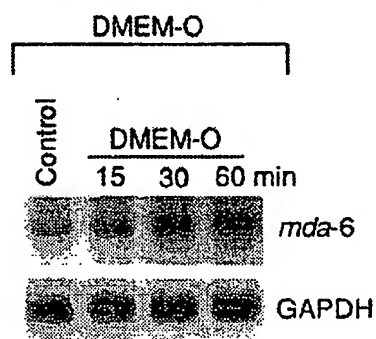
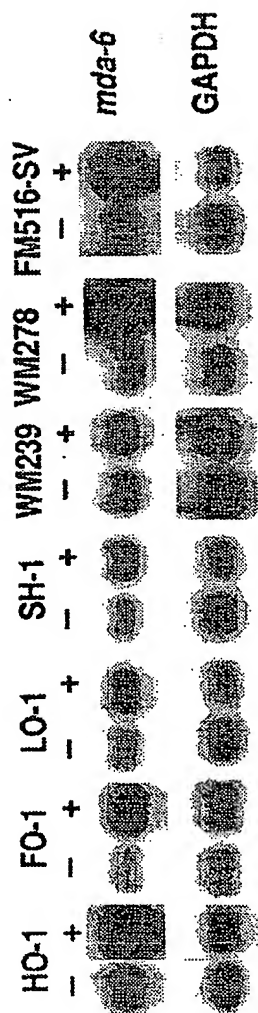


FIGURE 31E



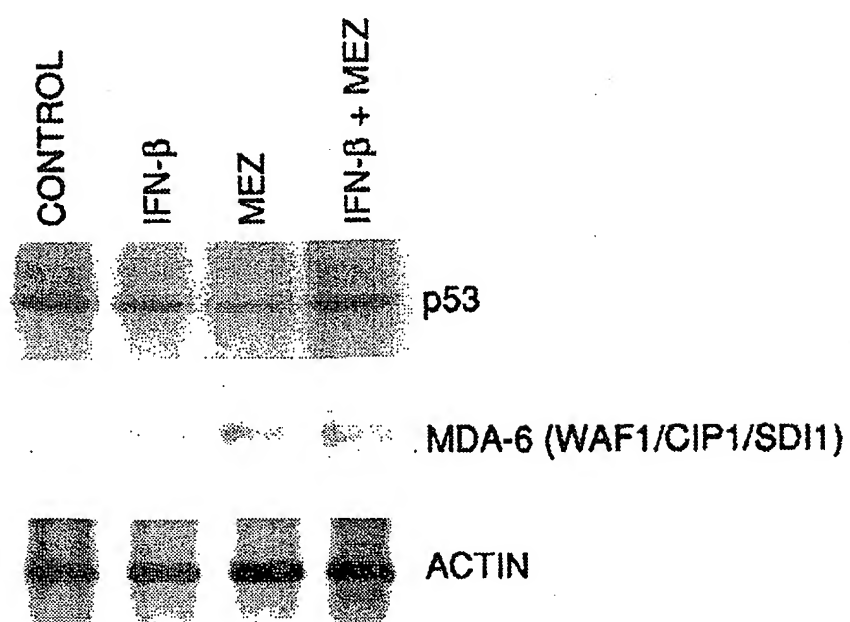
33/45

FIGURE 32



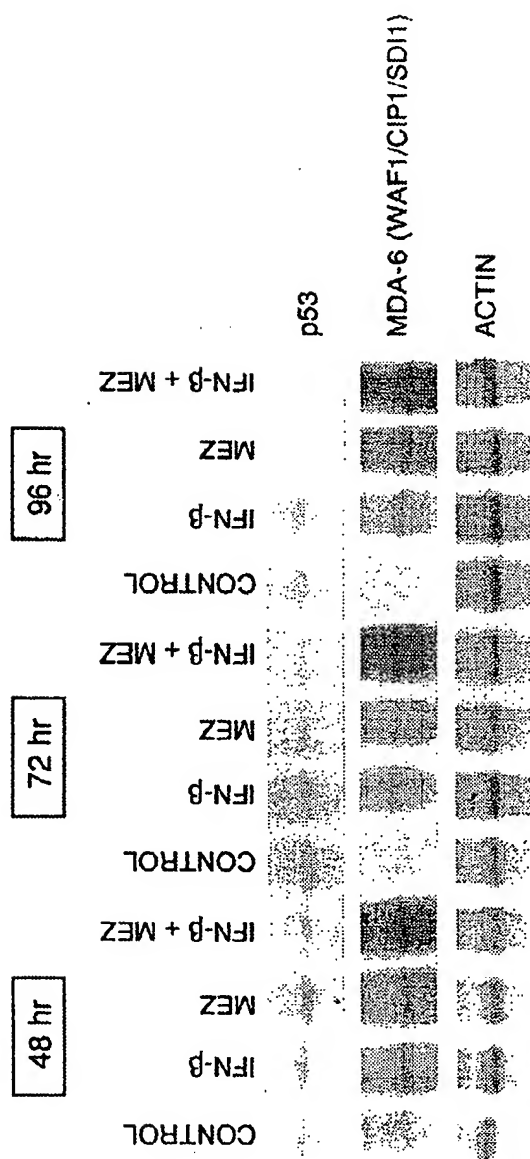
34/45

FIGURE 33



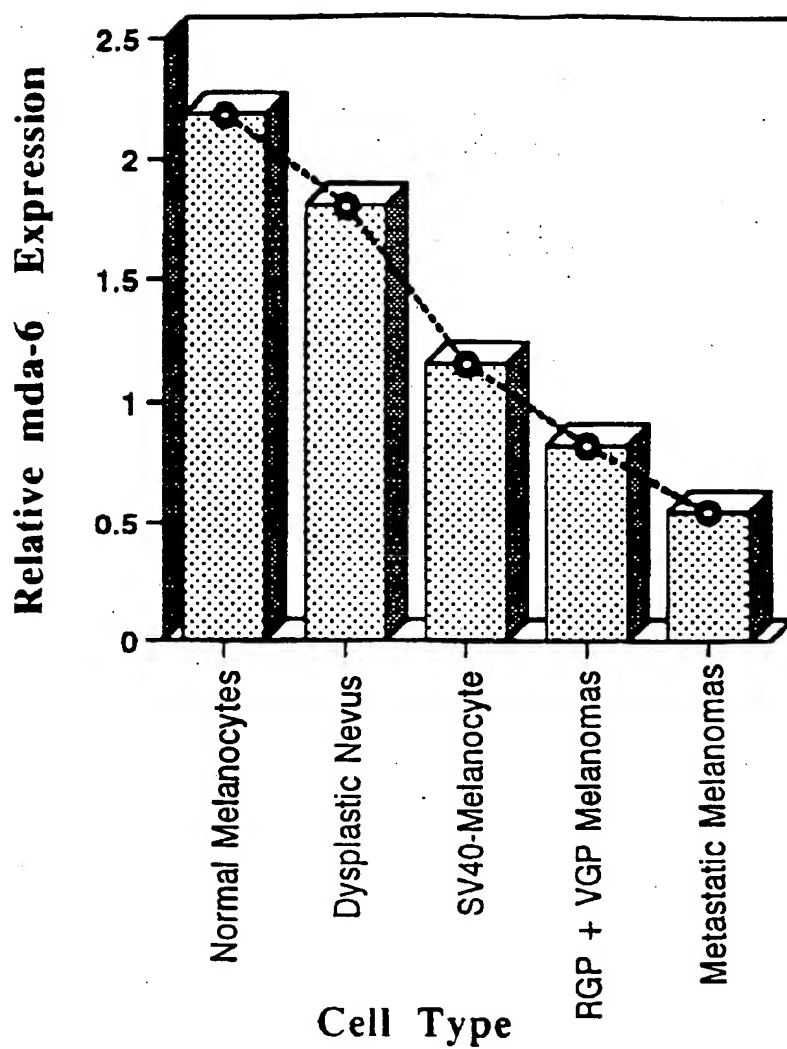
35/45

FIGURE 34



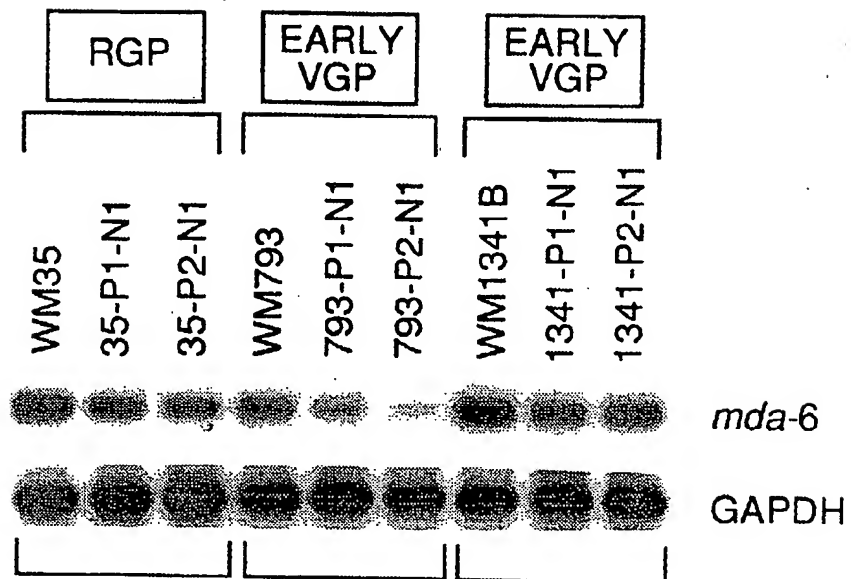
36/45

FIGURE 35



37/45

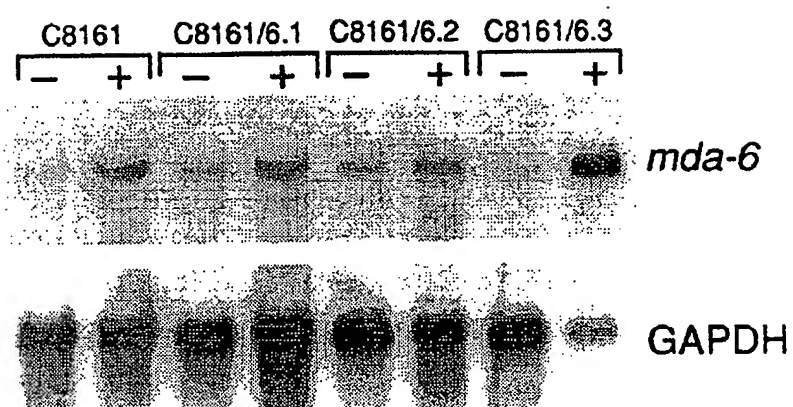
FIGURE 36





38/45

FIGURE 37



39/45

## FIGURE 38A

FIGURE 38A

FIGURE 38B

MDA-7.Seq

CTGGCCTGCAAAACCTTTACTTCTGAAATGACTTCCACGGCTGGACGGGAACCTTCCACC 60  
 CACAGCTATGCCCTCTGATTGGTGAATGGTGAAGGTGCTGTCTAATTTCTGTAAAGAAG 120  
 AACCAGCTGCCCTCCAGGCAGCCCTCAAGCATCACTTACAGGACCAGAGGGACAAGA 180  
 CATGACTGTGATGAGGAGCTGCTTTCGCCAATTAAACCAAGAAGAAATTGAGGCTGCTT 240  
 GGGAGGAAGGCCAGGAGGAACACGAGACTGAGAGATGAATTTTCAACAGAGGCTGCAAAG 300  
 M N F Q Q R L Q S

CCTGTGGACTTTAGCCAGACCCCTTCTGCCCTCCTTTGCTGGCGACAGCCTCTCAAATGCA 360  
 L W T L A R P F C P P L L A T A S Q M Q

GATGGTTGTGCTCCCTTGCCCTGGGTTTACCCCTGCTTCTCTGGAGCCAGGTATCAGGGGC 420  
 M V V L P C L G F T L L L W S Q V S G A

CCAGGGCCCAAGAATTCCACTTTGGGCCCTGCCAAGTGAAGGGGTTGTTCCCCAGAAACT 480  
 Q G Q E F H F G P C Q V K G V V P Q K L

GTGGGAAGCCTTCTGGGCTGTGAAGACACTATGCAAGCTCAGGATAACATCACGAGTGC 540  
 W E A F W A V K D T M Q A Q D N I T S A

CCGGCTGCTGCAGCAGGAGGTTCTGCAGAACGTCTCGGATGCTGAGAGCTGTTACCTTGT 600  
 R L L Q Q E V L Q N V S D A E S C Y L V

CCACACCCCTGCTGGAGTTCTACTTGAAAACTGTTTCAAAAACCTACCACAATAGAACAGT 660  
 H T L L E F Y L K T V F K N Y H N R T V

FIGURE 38B

TGAAGTCAGGACTCTGAAGTCATTCTCTACTCTGGCCAAACAACCTTGTCTCATCGTGTC 720  
E V R T L K S F S T L A N N F V L I V S

ACAACTGCCAACCCAGTCAAGAAAATGAGATGTTTCCATCAGAGACAGTGCCACACAGGCG 780  
Q L Q P S Q E N E M F S I R D S A H R R

GTTTCTGCTATTCCGGAGAGCATTCAAACAGTTGGACGCTAGAACGAGCAGCTCTGACCAAAGC 840  
F L L F R R A F K Q L D V E A A L T K A

CCTTGGGGAAGTGGACATTCTTCTGACCTGGATGCAGAAATTCTACAAGCTCTGAATGTC 900  
L G E V D I L L T W M Q K F Y K L \*

TAGACCAGGACCTCCCTCCCTGGCACTGGTTTGTTCCTGTGTCAATTTCAAAACAGTCT 960  
CCCTTCCATAGCTGTCTACCTGGACACTTCACGCCCTTGCCCATGGTCCCATTTCTTGGCC 1020  
CAGGATTAATTGTCAAAGAGTCATTCTTAAAGCAGCGCCAGTGACAGTCAGGGAAGTGTC 1080  
CTCTGGATGCTGTGAAGAGTCTACAGAGAAGATTCTTGTATTATTACAACTCTATTAA 1140  
TTAATGTCAGTATTTCAACTGAAGTTCTATTATTGTGAGACTGTAAGTTACATGAAGG 1200  
CAGCAGAATAATTGTGCCCCATGCTTCTTTACCCCTCACAAATCCTTGCCACAGTGTGGGC 1260  
AGTGGATGGTGCTTAGTAAGTAACTTAATAAAGTGTGCTTTTGTGGCTGTCTTTG 1320  
GATTGTTAAATAACAGAGAGGATGCTTGGATGTAAACCTGAACCTTCAGAGCATGAAAT 1380  
CACACTGCTGCTGATATCTGCAGGACAGAGCATTTGGGTGGGTAAAGTGATCTGT 1440  
TTGAAAAGTAAACGATAAAATGTGGATTAAAGTGCCACGACAAAGCAGATCCTCAATAA 1500  
ACATTTTCATTTCCACCCACACTCGCCAGCTCACCCCATCATCCCTTTCCCTTGGTGCCC 1560  
TCCTTTTTTTTTATCCTAGTCATTCTTCCCTAATCTTCCACTTGAGTGTCAAGCTGACC 1620  
TTGCTGATGGTGACATTGCACCTGGATGTACTATCCAAATCTGTGATGACATTCCTGCTA 1680  
ATAAAGACAACATAACTCAAAAATAAAATAAA 1718

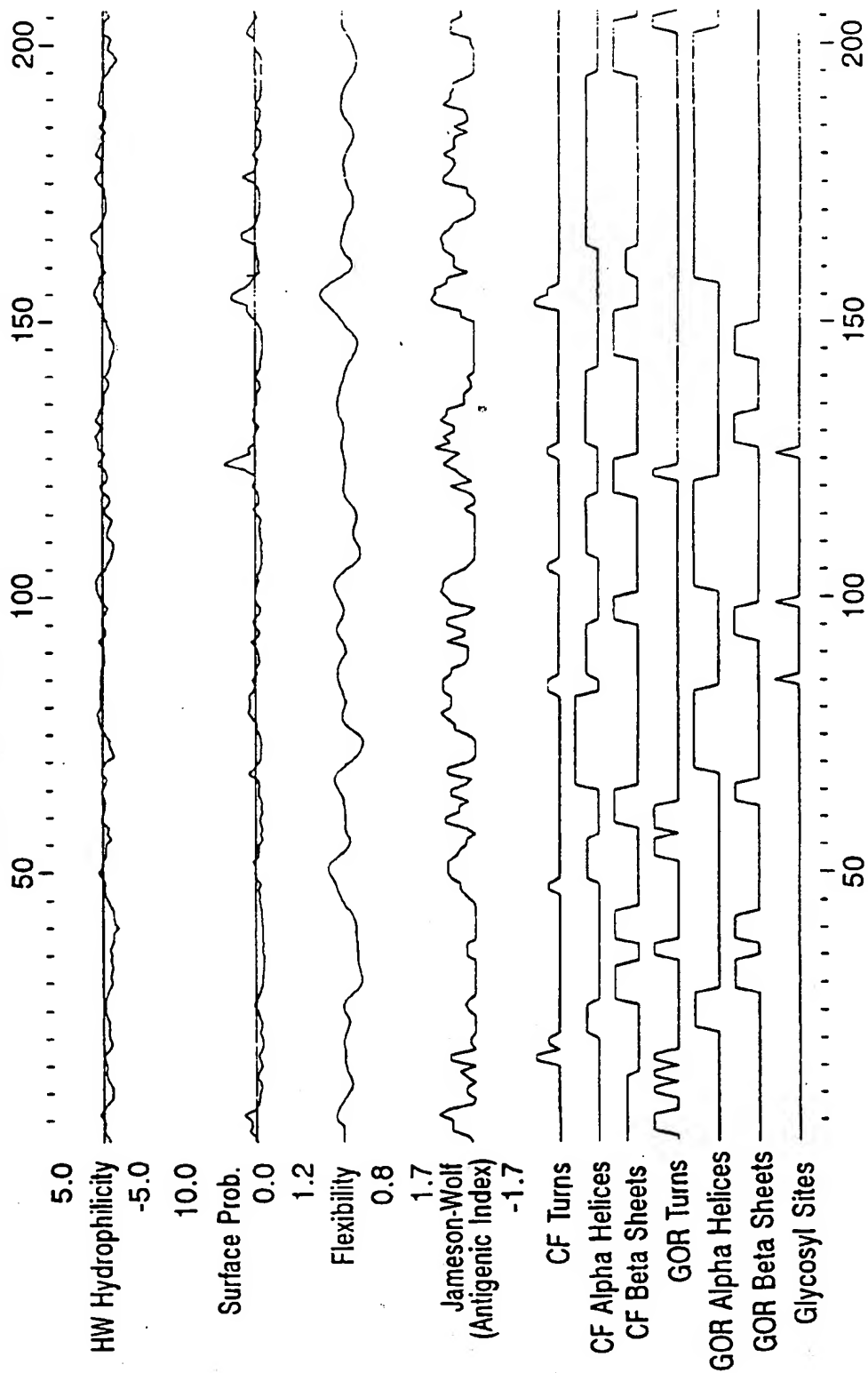
41/45

FIGURE 39

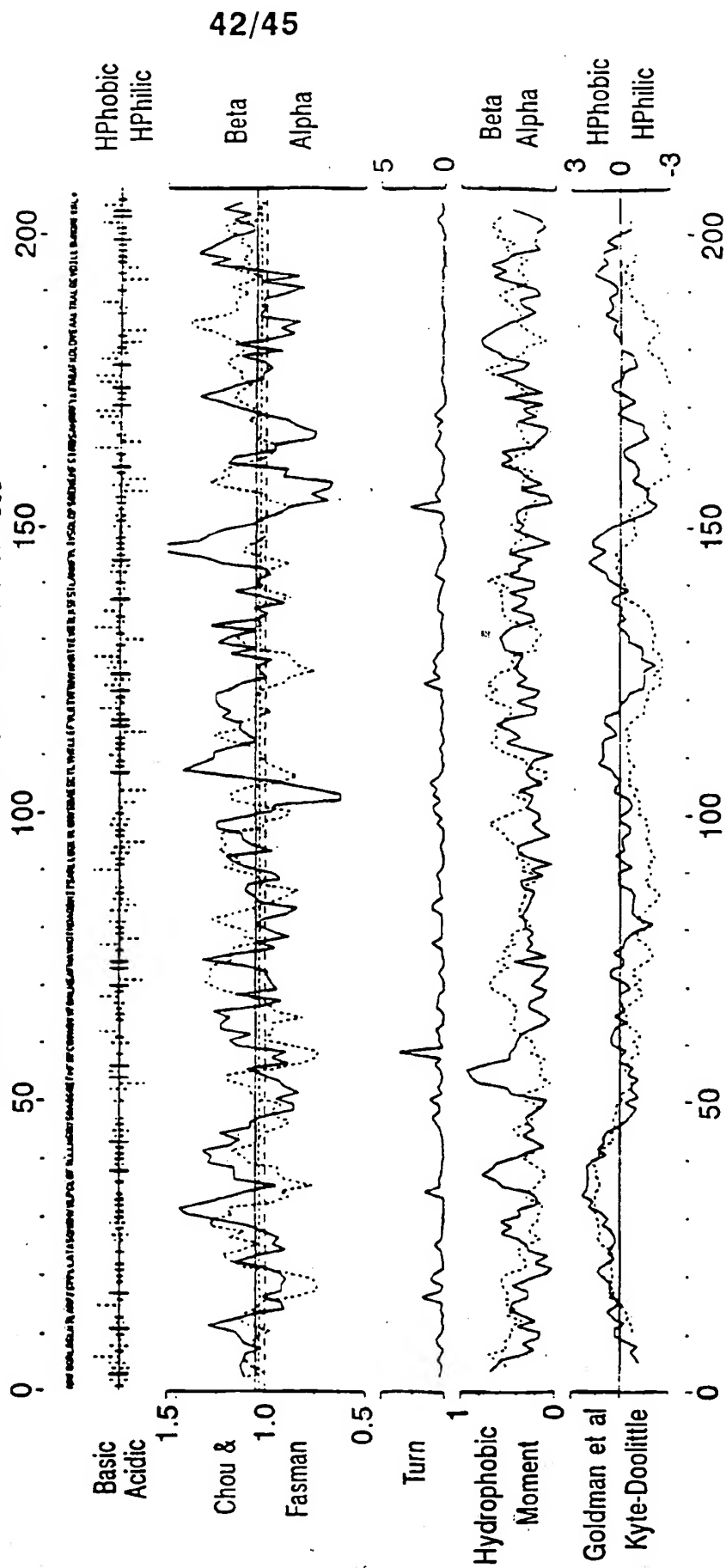
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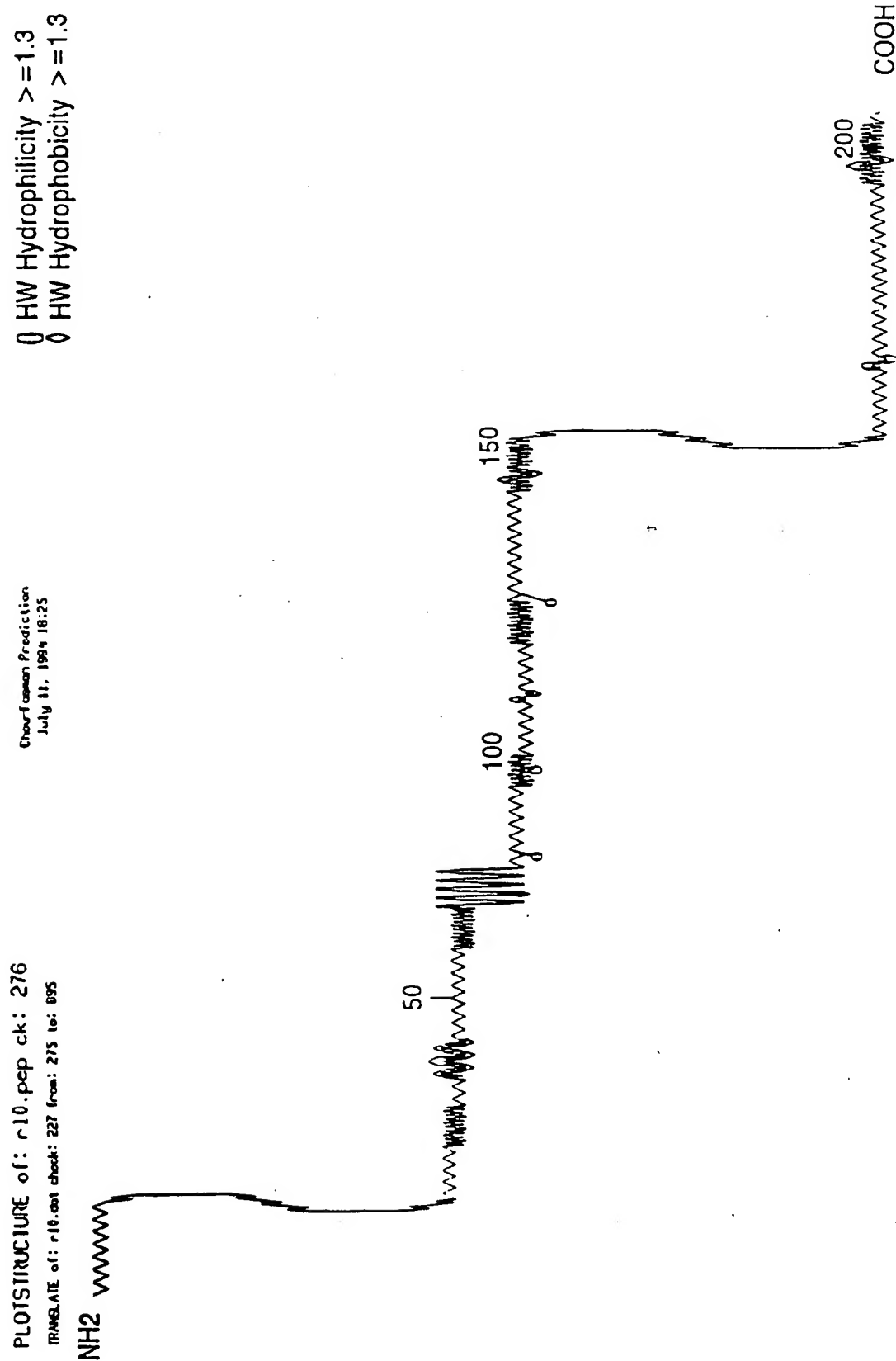


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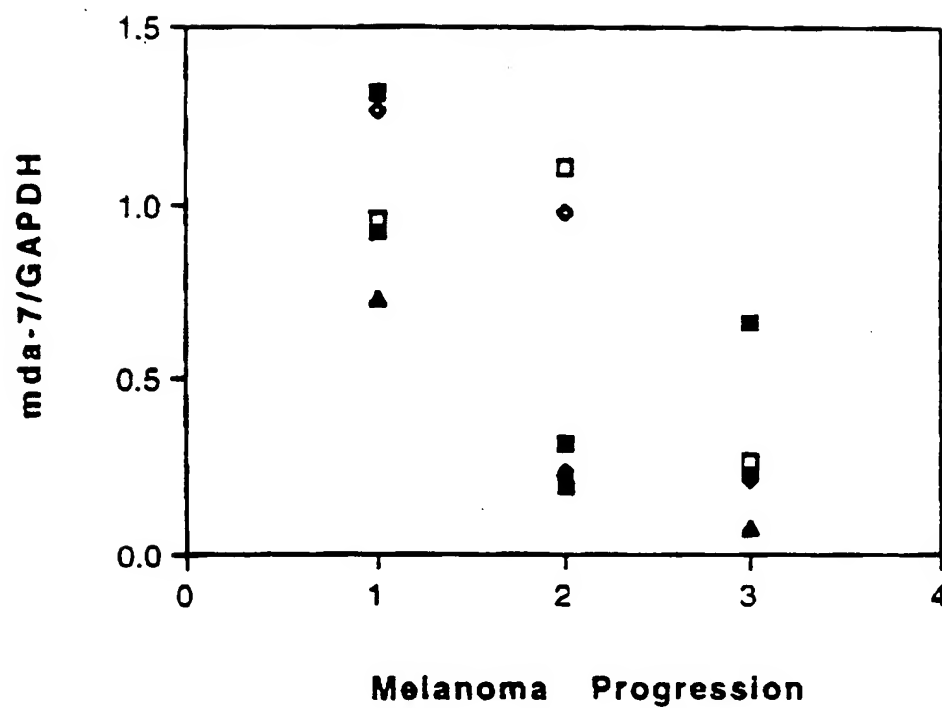
43/45

FIGURE 41



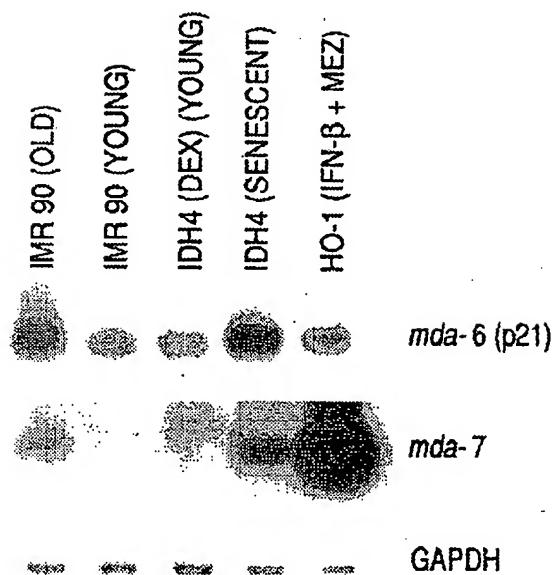
44/45

FIGURE 42



45/45

FIGURE 43





## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/12160

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C12P 19/34; C07H 21/04

US CL :435/91.2, 172.3; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/91.2, 172.3; 536/22.1,23.1,24.3,24.31,24.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, MEDLINE, WORLD PATENT INDEX, BIOTECH ABSTRACTS, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Analytical Biochemistry, Volume 214, issued 1993, Hara et al., "DNA-DNA Subtractive cDNA Cloning Using oligo(dT)30-Latex and PCR: Identification of Cellular Genes Which Are Overexpressed in Senescent Human Diploid Fibroblasts", pages 58-64, see especially the abstract and Figure 1 and Materials and Methods starting on page 59.	1,3,6,9-11, 14,19,22, 23 ----- 1-24
A	Nucleic Acids Research, Volume 19, Number 25, issued 1991, Hara et al., "Subtractive cDNA cloning using oligo(dT)30-latex and PCR: Isolation of cDNA clones specific to undifferentiated human embryonal carcinoma cells", pages 7097-7104, see especially the abstract, Figure 2 on page 7100, and the Materials & Methods starting on page 7098.	1-24

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

13 FEBRUARY 1995

Date of mailing of the international search report

24 FEB 1995

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 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ARDIN MARSCHEL

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/12160

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Nucleic Acids Research, Volume 18, Number 16, issued 1990, Rubenstein et al., "Subtractive hybridization system using single-stranded phagemids with directional inserts", pages 4833-4842, see especially the abstract and Materials and Methods starting on page 4833.	1,2,4,6-10, 22, and 23 ----- 1-24
A	Maniatis et al, "MOLECULAR CLONING, A LABORATORY MANUAL", published 1982 by Cold Spring Harbor Laboratory, (Cold Spring Harbor, New York), pages 224-228, see entire disclosure on pages 224-228.	1-24
X --- Y	Proceedings of the National Academy of Sciences (USA), Volume 85, issued March 1988, Travis et al., "Phenol emulsion-enhanced DNA-driven subtractive cDNA cloning: Isolation of low-abundance monkey cortex-specific mRNAs", pages 1696-1700, see especially the abstract Materials and Methods starting on page 1696.	1,2,4,6,9, 10,23 ----- 1-24
X --- Y	Proceedings of the National Academy of Sciences (USA), Volume 85, issued August 1988, Duguid et al., "Isolation of cDNAs of scrapie-modulated RNAs by subtractive hybridization of a cDNA library", pages 5738-5742, see especially the abstract and Materials and Methods starting on page 5738 and more especially Figure 1 on page 5739.	1,2,4-7,9, 10,14, 19-23 ----- 1-24

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/12160

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-24

Remark on Protest

☐  
☐

- The additional search fees were accompanied by the applicant's protest.  
No protest accompanied the payment of additional search fees.

International application No.  
PCT/US94/12160

**This ISA found multiple inventions as follows:**

The inventions listed as Groups I-XVII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The invention of Group I is independent of Groups II-XVII because Group I is directed to cDNA library generation and the library generated without any limitation directed to mda genes or methods directed to mda genes and therefore does not share a special technical feature with any of Groups II-XVII. Similarly, the invention of Group XVII lacks a common technical feature with the other Groups because it is directed to a hybridization detection method using generically cited probes that are specific to any mda gene. Therefore there is no special technical feature that links this method to any of the specific mda genes of Groups II-XVI. Regarding Groups II-XVI, the following Groupings are independent inventions that lack a common technical feature because of their being directed to different, specific, and independent mda genes:

### Additionally, Groups

In summary, Groups I-XVII lack a common linking special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept and therefore support a lack of unity between these groupings of claims.